Improving microalgae for biofuel production

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Abstract

Microalgae are a diverse group of oxygenic photosynthetic microorganisms which show great promise as a source of biofuel. However, significant challenges still remain before microalgae can be considered a viable source of biofuel. The main current challenges are nutrient sourcing and recycling as well as downstream processing. The algal cell wall and especially the presence of an algaenan cell wall in some Chlorophyte algae could be an important variable in determining downstream processing costs but not much comparative research has been done to elucidate this. The first part of the present study focuses on the recently isolated alga *Pseudochoricystis ellipsoidea* (Trebouxiophyceae) and its improvement and assessment for biofuel production. Random mutagenesis and FACS screening protocols were developed for the isolation of pigment and cell wall mutants but despite considerable efforts no suitable mutants could be identified in the first half of this project. Two 500 L raceway ponds as well as an algal growth room and bubble column bioreactors were set up to facilitate algal research at the University of Bath and assess the performance of *P. ellipsoidea* in realistic culture conditions. *P. ellipsoidea* showed a maximum growth of 1.53 divisions day^{-1} in semi-open raceway ponds, resistance to contamination and a 30% lipid content, making it particularly suitable for raceway pond cultures. In the second part of this project six species of Chlorophyte ("green") algae, three of which produced algaenan, were compared for suitability to growth in anaerobic digestate and municipal wastewater as well as cell wall strength, permeability and suitability to hydrothermal liquefaction. We found that anaerobic digestate was a good medium for the growth of all species independently of autoclaving and that non-autoclaved wastewater was a very challenging medium. Algaenan production did not affect cell disruption by ultrasonication but growth stage and cell wall thickness did. Lipid extraction kinetics by chloroform/methanol were greatly affected by algaenan, meaning that this material is relatively impermeable to organic solvents. Cell wall thickness, cell volume and lipid content also had an effect on lipid extraction kinetics but this was only measurable after 180 minutes of extraction.

Hydrothermal liquefaction showed high solid and low oil yields, very low sulphur (≤0.1 %) as well as a 1.1 % -1.8 % nitrogen content which is significantly lower than most algal HTL studies to date. This suggests that stationary stage algae are more difficult to process but give a cleaner biocrude and reduce the loss of nitrogen through incorporation in the oil. Significant opportunities for optimisation still exist in the HTL process.

Abbreviations

AD – anaerobic digestate

ADM – anaerobic digestate media

AU – arbitrary units

BBM – Bold's Basal Medium

BODIPY $^{493/503}$ - (4,4-difluoro-1,3,5,7,8-pentamethyl-4-bora-3a,4a-diaza-s-indacene)

CCAP – Culture Collection of Algae and Protozoa

CE – *Chlorella emersonii* strain CCAP 211/8p

CF – *Chlorella* sp. Strain FC2-IITG

CV – *Chlorella vulgaris* strain CCAP 211/11b

CM – *Chlorella vulgaris* var. "*minutissima*" strain CCAP 211/52

CZ – *Chlorella zofingiensis* strain CCAP 211/51

dH_2O – deionised water

ddH_2O – deionised and distilled water

DMSO – dimethylsulfoxide

DPM – DENSO Pond Medium

DW – dry weight

EMS – ethyl methanesulphonate

FACS – fluorescence activated cell sorter

FAME – fatty acid methyl ester

FSC – forward scatter

GM – genetic modification/ genetically modified

HTL – hydrothermal liquefaction

OD – optical density

PAR – Photosynthetically Active Radiation

PBR - Photobioreactor

PE – *Pseudochoricystis ellipsoidea* strain Ni

sL – standard litres (for gas flows)

TAG - triacylglyceride

TEM – transmission electron microscopy

TSS – total suspended solids

UV – ultraviolet

VLCFA – very long chain fatty acids

WW – waste water

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1 Introduction

1.1 Potential microalgal solutions to 21st century problems.

The extensive usage of fossil carbon sources such as coal, oil and gas as energy sources over the past two centuries has revolutionised human society in most parts of the planet propelling humanity into the industrial age. However these energy sources are both rapidly depleted and rapidly increasing CO_2 concentrations in the atmosphere (IPCC, 2007). Sustainable energy sources are thus needed to minimise the impact of humanity on the carbon balance of the planet and to provide energy for a post-fossil fuel era. Many different sustainable energy sources are currently undergoing intensive research and development (solar, wind, nuclear fusion, biogas generation to name a few) however liquid hydrocarbon fuels will always be in demand, expecially by the aviation industry due to their high energy density and practicality (Williams and Laurens, 2010). Biodiesel derived from palm oil, soybean oil and canola seed oil among others as well as bioethanol derived from the fermentation of maize have seen an increase in production over the past few decades as a way for governments to meet targets for reducing carbon emissions and renewable fuel. However these require fertile land which has to be allocated either at the expense of food or at the expense of pristine land (Gallagher, 2008).

Algae is a term that encompasses a huge diversity of essentially oxygenic photosynthetic organisms that can be prokaryotes (cyanobacteria) or eukaryotes and have arisen from single or multiple endosymbiotic events (Knoll, 1992, 2014). Algae can be single celled, colonial or macroscopic (seaweeds), are primarily aquatic but can be found in most environments and conditions, even deserts (Leliaert *et al.*, 2012). The term microalgae refers to the microscopic, single celled or colonial forms of algae but in this work the terms algae and microalgae are used interchangeably and always refer to the microscopic, single celled species. Microalgae grow by using light energy to reduce carbon dioxide to glucose in a process that splits water and releases

oxygen. They and their terrestrial plant descendants are the only source of oxygen and the main source of energy utilisable by other life-forms on this planet and thus are the basis of almost all life on earth and certainly all multicellular life (Stephenson *et al.*, 2011).

The industrial culturing of microalgae is a potential solution to the production of liquid transport fuels because microalgae have a number of desirable characteristics. Microalgae fix atmospheric CO_2 (Packer, 2009), are at least 5-15 times more productive per unit land area than plant biofuels (Clarens *et al.*, 2011), they do not require arable land and produce lipids as energy storage molecules that can be readily converted for use in current internal combustion engines (Scott *et al.*, 2010; Williams and Laurens, 2010). Algae can also produce a range of valuable by-products such as protein for animal feed, polyunsaturated fatty acids (especially omega-3 oils), antioxidants, bioactive compounds and other nutraceuticals and pharmaceuticals (Christaki *et al.*, 2011; Milledge, 2010).

1.2 Current challenges to the commercialisation of microalgae.

1.2.1 Cost and reliability of industrial growth systems.

Much research has focused on the growing technology in the past decade with open, flattened toroidal (raceway) ponds mixed by a simple paddlewheel, being the standard way of growing algae (Chaumont, 1993) and being compared to numerous designs of closed photobioreactors which feature higher volumetric productivity, better land efficiency and better utilisation of light than ponds (Jorquera *et al.*, 2010) as well as a reduction of contamination due to their closed design (Wang et al., 2013),. Algal biofuels derived from photobioreactor cultures are generally more energy intensive (Jorquera *et al.*, 2010; Stephenson *et al.*, 2010), more expensive (Amer *et al.*, 2011) and emit more CO_2 (Stephenson *et al.*, 2010) as well as being less reliable due to the more complex design, higher number of parts and potential issues with biofouling (Arbib *et al.*, 2013).

It is now generally accepted that for such a low-cost product as biofuel, earthen ponds, sometimes sealed with clay, are the only cost-effective solution with closed bioreactor systems being investigated mainly for the production of high-value products (Chaumont, 1993; Jorquera *et al.*, 2010). So while algae have great potential in terms of productivity and use of marginal land, the technology is still immature and suffers from a low energy return on investment (EROI) (Liu *et al.*, 2013) and high price (Amer *et al.*, 2011). Currently, the main hurdles to the viability of algal biofuels are; the high energetic burden of inorganic fertilisers needed in most cases to support algal growth, the high cost of supplying CO_2, needed to enhance algal growth and the hugely energy intensive downstream steps required to dewater and extract lipid from the algal biomass.

1.2.2 Nutrient recycling and the use of wastewaters as "free" nutrients to supplement algal biofuel plants.

Life cycle studies show nutrient demand and especially the need for inorganic ammonia fertiliser to be one of the main energetic burdens of algal biofuel production (Clarens *et al.*, 2010; Lardon *et al.*, 2009; Liu *et al.*, 2013). This is mainly because algae need more fertiliser than land plants per unit of oil produced (Lam and Lee, 2012). Clarens *et al.* (2010) highlighted the fact that using wastewater as a nutrient source could offset some of the energetic burden associated with algal biomass production. Some life-cycle studies have reported favourable energy return on investment (EROI) ratios for coupling wastewater treatment to algal biofuel production (Beal *et al.*, 2012; Sturm and Lamer, 2011) and some large-scale systems have been set up to couple these two processes (Christenson and Sims, 2012; Craggs *et al.*, 2012) which have yielded encouraging results both in terms of algal growth and wastewater bioremediation. However it will not always be possible to couple algal biofuel production to wastewater bioremediation on an industrial scale. This is because wastewater treatment plants (WWTPs) are usually located in the vicinity of urban areas and the relatively large land footprint of algal

raceway ponds means that it will not always be possible or economically feasible to acquire the required land for the installation of an algae biofuel plant in the vicinity of a WWTP. Furthermore locating an algal biofuel plant on urban land or farmland would eliminate one of the major advantages of algal biofuels, which is their ability to utilise non-arable land.

Anaerobic digestate has also been proposed as a waste nutrient source for algal growth (Olguín, 2012) due to its satisfactory N/P ratio and the presence of bicarbonate and organic acids that could support mixotrophic growth of algae and thus reduce the need for CO_2 bubbling which is one of the main costs of microalgal culturing (Lardon *et al.*, 2009). Recent studies have found that anaerobic digestate, mainly in the form of digested dairy and pig manure, is a satisfactory medium for microalgae culturing as long as the dilution is optimised (Uggetti *et al.*, 2014a; Wang *et al.*, 2010). Magnesium has been found to be a possible limiting factor when digested manure serves as the nutrient source but this was found to be amended by co-supplying algal digestate which provided enough magnesium to support algal growth (Bjornsson *et al.*, 2013).

Finally, some authors have highlighted the potential of recycling nutrients from the algal biomass as a growth medium. This would create a semi-closed loop where most of the nutrients originate from the final processing step of the extracted biomass thus greatly reducing the need of nutrient import whether this is in the form of inorganic fertilisers or waste nutrients. In particular anaerobic digestion of algal biomass and recycling of the digestate has been proposed as a closed nutrient loop (Erkelens *et al.*, 2014; Ward *et al.*, 2014) and the aqueous phase of hydrothermal liquefaction of algal biomass has also been shown to be a suitable growth medium for algae (Biller *et al.*, 2012). Both above systems also provide a gaseous phase rich in CO_2, so the potential for a closed loop in the cycling of most elements is considerable.

Thus the emerging paradigm for algal biomass production is that of an integrated approach where most of the nutrients are recyclable and limited

amounts of "waste" nutrients are imported to supplement for inherent systematic losses. This scenario will hopefully lead to a sustainable process with good energy and economic balance as well as minimum environmental impact.

1.2.3 Two emerging downstream pathways to algal biofuels: merits and challenges.

While the upstream challenges for optimising algal growth are relatively straightforward, major advances in downstream processing are still necessary to make algal biofuels a reality. There are several downstream factors currently preventing the commercialisation of algal fuel. Water content at harvest will be at best around 99.7 % (w/w) if grown in bioreactors (Olofsson *et al.*, 2012) but more realistically around 99.95 % - 99.98 % (w/w) if grown in open ponds (Craggs *et al.*, 2012). Dewatering of the algae biomass and especially the final drying step are very energy intensive (Uduman *et al.*, 2010). It is, therefore, likely that any industrial extraction process will have to tolerate an approximately 85 % water content in the biomass (Cooney *et al.*, 2009). Cell disruption currently seems to be an important processing step to enhance lipid recovery because algae generally have tougher cell walls than plants and their cell walls can be impermeable to solvents (Lee *et al.*, 2012, 2010; Prabakaran and Ravindran, 2011) but so far no commercial scale disruption method has been demonstrated. Finally, it is still unclear what process and combination of solvents would be optimal for extracting lipids from microalgae and also how species selection would impact lipid extraction.

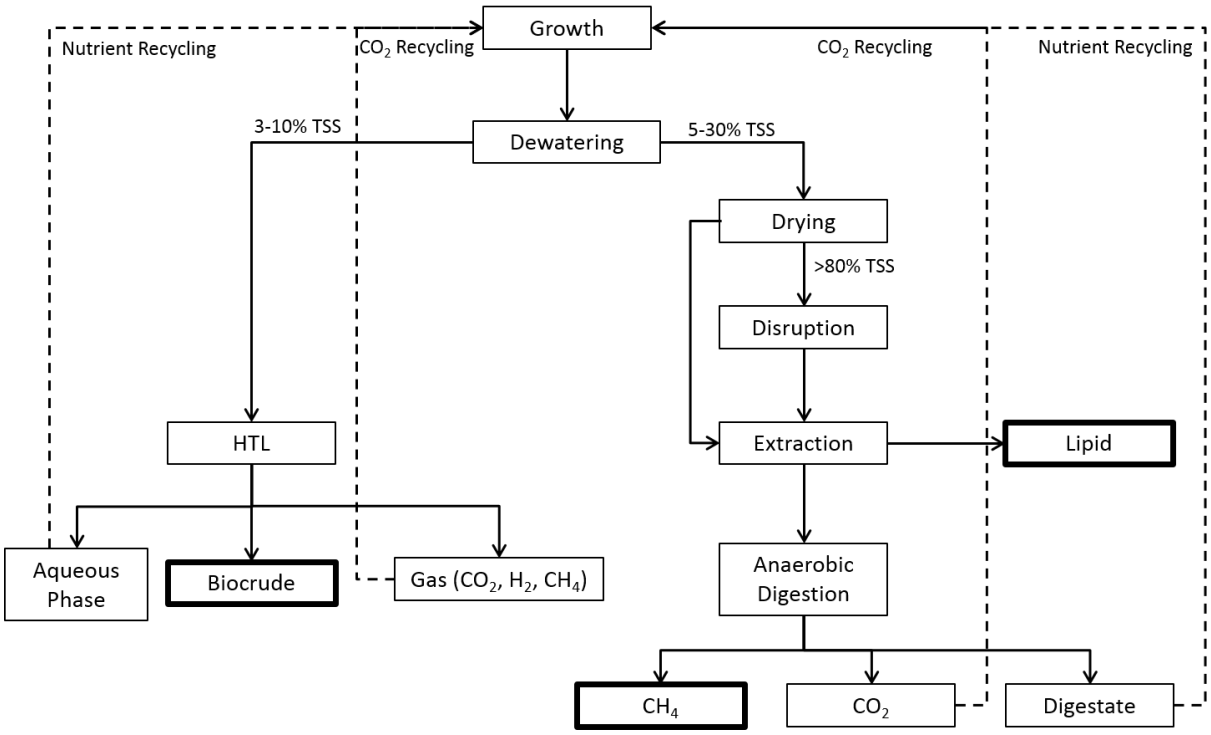

Figure 1.1. Conceptual outline of two realistic future scenarios for algal biofuels in fully integrated, zero waste pathways; hydrothermal liquefaction (HTL) on the left and the cell disruption/lipid extraction coupled with anaerobic digestion on the right. Thick rectangles represent system outputs and dashed lines represent recycling of nutrients and CO_2.

These considerations have led to the recent development of, broadly speaking, two paradigms for the downstream fate of algal biomass; the biorefinery approach (Gonzalez-Delgado and Kafarov, 2011; Hariskos and Posten, 2014) and hydrothermal liquefaction (Barreiro *et al.*, 2013a; Frank *et al.*, 2013) (fig. 1.1.).

1.2.4 Hydrothermal liquefaction.

Hydrothermal liquefaction (HTL) is a process that eliminates the need for extensive dewatering, cell disruption and lipid extraction by transforming the whole wet biomass (≈85% water) into a bio-crude oil using sub-critical water (Barreiro *et al.*, 2013a). By using wet biomass, dewatering and drying are not necessary, furthermore the high temperatures (280-370 °C) and pressures (10-25 MPa) used mean that cell disruption is not necessary as the whole biomass effectively breaks down, water becomes less polar and organic

compounds become more soluble, eliminating the need for an organic solvent (Cooney *et al.*, 2009). The exact mechanism of HTL has not been fully elucidated but it is thought that biomass undergoes rapid hydrolysis to highly reactive monomers which then polymerise to form biocrude oil, gas and solid compounds (Barreiro *et al.*, 2013a). The end-product consists of an oil phase similar to a heavy biocrude (Ross *et al.*, 2010), a gaseous phase containing CO_2 (which can be recycled for algal growth), H_2 and CH_4 (which can be used as fuels in their own accord (Liu *et al.*, 2013)), a solid phase which contains mainly ash and an aqueous phase which contains most nutrients along with dissolved organic carbon. The aqueous phase can be diluted and recycled as an algal growth medium (Biller *et al.*, 2012). HTL and lipid extraction seem to be at least comparable in their energy balance (Frank *et al.*, 2013), however HTL seems to be recently favoured because it eliminates all the major current hurdles of downstream processing (dewatering, disruption, extraction) in a single step and because it has been impressively quick to scale up to pilot-scale (Jazrawi *et al.*, 2013; Liu *et al.*, 2013). Indeed the only company currently able to produce algal liquid fuel on a reasonably large scale is Sapphire Energy (New Mexico, USA) who use HTL to transform biomass to fuel and likewise the only life-cycle analysis based entirely on real data has been published by Liu *et al.* (2013) based on data from Sapphire's pilot facilities. The main problem with HTL is the high nitrogen content of the biocrude, which in the literature ranges between 3.2 % and 7.1 % (Barreiro *et al.*, 2013a). This results both in high NO_x emissions when combusted but also represents a loss of nitrogen from the nutrient cycling system which is very costly, at least when replaced with conventional fertilisers (Liu *et al.*, 2013). Furthermore it means that HTL oil cannot be used directly as a transportation fuel without upgrading (Barreiro *et al.*, 2013b). Finally, it has recently been shown (Barreiro *et al.*, 2013b) that HTL gives species-dependent crude oils but when the temperature of the process was increased from 250 °C to 375 °C, these differences became less significant. This suggests that HTL can give similar results for most species and undefined consortia of algae and other microorganisms.

1.2.5 The biorefinery approach.

The biorefinery approach basically seeks to extract as many different products as possible from the algal biomass for the highest market value possible. The same approach is taken by oil refineries where the bulk of the product may be fuel but the bulk of the profit comes from specialty chemicals. Pigments, neutraceuticals, pharmaceuticals, bio-active compounds and animal feed are among the products that can be produced by algae (Milledge, 2010). The extracted biomass (if there is an end-point where biomass remains) can be subjected to anaerobic digestion in order to obtain methane and digestate to be used as an algal growth medium in subsequent growing cycles (Morken *et al.*, 2013; Uggetti *et al.*, 2014b). While a great number of different scenarios are possible depending on the species of algae used and the value-added products that can be obtained from them, the most often cited scenario is that lipids would be extracted by solvents such as hexane, heptane or chloroform/methanol (Cooney *et al.*, 2009; Halim *et al.*, 2012) with cell disruption preceding lipid extraction if necessary.

Organic solvent extraction of lipid from microalgal cells is based on the chemical principles of "like dissolving like" and has been shown to follow first order kinetics (Halim *et al.*, 2011) described by the equation:

$$Y_t = Y_m[1 - e^{-tk}] \quad (1)$$

where Y_t is the lipid yield (weight of lipid extracted/weight of dry biomass) for a given extraction time (t), Y_m is the maximum lipid yield of the extraction (g lipid / g dry biomass) and k is the lipid mass transfer coefficient from the algal cells to the solvent (min^{-1}). Both Y_m and k vary as a function of several variables: Y_m varies with the type of solvent, the actual lipid content of the algal biomass and with R, the ratio of solvent (ml) to dried algal biomass (g). The higher R is the closer the lipid yield is to the total lipid content of the algae (McConnell and Farag, 2013). The mass transfer coefficient is a function of several variables many of which are relatively unexplored. Current knowledge in lipid extraction methods and operating

variables has been comprehensively reviewed by Halim *et al.* (2012), who state that the mass transfer coefficient is a function of agitation speed, solvent to mass ratio (R) and extraction temperature.

Some form of cell disruption will likely be essential in species with a particularly hard/resistant cell wall as this has been shown to limit the extractability of lipids (Ranjan *et al.*, 2010) and the digestibility of animal feed (Milledge, 2010) in some cases. Ward *et al.* (2014) reviewed the current state of algal anaerobic digestion and concluded that resistant cell wall materials such as algaenan limit methane yields in anaerobic digestion. Lee *et al.* (2012) have assessed current disruption methods and found that the disruption step alone would require more energy than that present in the biomass but also that these methods were highly inefficient, consuming more than 100 times the theoretical energy required for disruption. Therefore significant potential for progress exists in optimising cell disruption.

The need and extent of dewatering, the necessity and immaturity of cell disruption technologies and the complexity of multiple product extraction as well as scalability issues of the above are the main hurdles currently imposed on adopting this approach and have been comprehensively reviewed by Cooney *et al.* (2009). In fact, downstream processing of algae in the above manner (solvent extraction, sometimes preceded by cell disruption) is in such an immature stage that most life-cycle analyses assessing algal biofuels have to; either ignore the downstream processing (Clarens *et al.*, 2010), assume that the cost will eventually be the same as soybean or other oilseed extraction (Clarens *et al.*, 2011; Collet *et al.*, 2011; Lardon *et al.*, 2009; Yang *et al.*, 2010) or extrapolate from lab scale results with questionable scalability (Campbell *et al.*, 2010; Frank *et al.*, 2013; Stephenson *et al.*, 2010). Most studies on cell disruption and lipid extraction to date have focused on process variables and not much light has been shed on the biological variability of microalgae and the effect of this variability on the downstream processing costs and requirements.

1.2.6 *Biological variability and the potential role of the cell wall in assessing the suitability of algal species for cell disruption and lipid extraction.*

Inter-species variability in lipid extraction and cell disruption kinetics has, so far, been unexplored to our knowledge. Indeed cellular characteristics such as the presence and thickness of a cell wall, the composition of the cell wall, cell size and lipid content might affect the relative cost of lipid extraction and cell disruption. Since the cell wall is the main barrier for the diffusion and transport of all molecules in and out of the cell, it is likely that the cell wall will be the main rate-limiting barrier to the diffusion of solvent and lipid-solvent complexes (Halim *et al.*, 2012). Thus if the cell wall of a species is relatively impermeable to organic solvents, cell disruption becomes more essential as a means of increasing the extractability of algal lipids and other products.

Algae vary greatly on their cell wall composition from various combinations of polysaccharide-derived materials such as cellulose, mannose and glucosamine in the chlorophyte algae, organic plates in the dinoflagellates, silica frustules in the diatoms and algaenans (VLCFA-derived biopolyesters) in some chlorophytes (Kodner *et al.*, 2009) as well as some eustigmatophytes (esp. *Nannochloropsis* spp.) (Gelin *et al.*, 1997) and even some cyanobacteria (Biller, 2012; personal communication) (see section 1.5). It is conceivable that these different cell wall materials have different permeabilities to organic solvents and therefore would affect lipid extraction kinetics. It is also conceivable that different cells wall materials require different amounts of energy to be disrupted.

The case of algaenan cell walls within the green algae is especially interesting. Most Chlorophyta produce polysaccharide cell walls but some produce algaenan cell walls (Kodner *et al.*, 2009). Algaenan is a very long chain fatty acid derived biopolyester (Allard *et al.*, 2002; Blokker *et al.*, 2006) that has been noted for its extreme chemical stability and is hypothesised to act as a desiccation and infection barrier (Kodner *et al.*, 2009). The ecophysiological properties of algaenan are relatively unexplored, however

because many of the most promising microalgal species for biofuel production (*Nannochloropsis* spp., *Chlorella minutissima* and most *Scenedesmus* spp. to name a few) produce algaenan cell walls, it is important to assess the impact of using algaenan-producing species on downstream processes in the biofuel industry to optimise species selection and downstream operating conditions.

Apart from the cell wall composition, it is conceptually possible that cell wall thickness, cell size and lipid content also play a role in cell disruption and lipid extraction kinetics; if the cell wall is indeed the rate limiting barrier for solvent diffusion it is likely that a thicker cell wall would result in slower lipid extraction. Cell size could be important because the larger a cell becomes, the less surface area will surround a given amount of lipid (for a given cellular shape) and in the same way as the lipid content of a cell becomes higher more lipid is contained within a given surface area.

1.3 Improving microalgae for biofuel production.

1.3.1 The case for microalgal genetic engineering.

Considerable interest has been placed on genetically modifying algae to enhance their potential as biofuel producers. This has focused, broadly speaking in two categories; modifying lipids and associated pathways and modifying the light harvesting system of microalgae.

Much interest is placed on modifying lipid synthesis with the ultimate aim of increasing lipid production or modifying the produced lipids to be more suitable for biofuel production (comprehensively reviewed by Radakovits *et al.*, 2010). This can be done either by engineering algae to lower their capacity for storage of polysaccharides such as starch (Li *et al.*, 2010) or by overexpressing vital genes in the lipid synthesis pathway. The modification of lipid chain lengths, production of drop-in fuels or specific high-value hydrocarbons is also a topic of considerable interest within the field of microalgal GM. The molecules of interest in algae are the neutral lipids that serve as a long-term energy storage for algae and are present in the form of triacylglycerides (TAGs). TAGs generally require transesterification into fatty-acid methyl esters (FAMEs) before they can be used in vehicles. Engineering algae to produce straight-chain hydrocarbons, short chain alcohols or other molecules that can be used in diesel or jet engines without modification therefore make the process to algal biofuels more efficient by eliminating a whole processing step. Shortening the average chain length of algae by increasing the production of C:12 and C:14 fatty acids has been achieved and would lead to improved biodiesel characteristics (Radakovits *et al.*, 2011). Some success has also been achieved in increasing the yield of food supplements such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) in *Pavlova lutheri* using random mutagenesis (Meireles *et al.*, 2003) as well as astaxanthin production in *Haematococcus pluvialis* (Steinbrenner and Sandmann, 2006; Tripathi *et al.*, 2001).

The second major focus of genetic modification in microalgae is the optimisation of light harvesting and solar-to-biomass conversion efficiency in microalgae. Theoretical maxima for photosynthetic efficiency have been calculated at ≈ 8% - 10%. However these theoretical maxima are rarely realised with the highest photosynthetic efficiency reported in lab cultures of green microalgae being 3% and those of C3 plants being ≤ 0.1% (Melis, 2009). This is mainly due to the excessive absorption of light by the chlorophyll molecules assembled in the light harvesting antennae of photosystems I and II, especially in high light conditions where 80% of photons absorbed can be dissipated as autofluorescence or non-photochemical quenching (NPQ) (Melis *et al.*, 1998). Thus a reduction in the number of chlorophyll molecules that make up the light harvesting antennae would eliminate wasteful absorption of light by the top layer of cells in a culture, reduce photoinhibition and allow for a longer functional light path to be used in algal culturing systems. The creation of truncated light harvesting antenna (tla) algal strain has, therefore, the potential of increasing productivity per unit area by up to 300% (Melis, 2009 and references therein). A *Chlamydomonas reinhartdii* tla mutant transformed with a linearised plasmid has been reported to have a 45% higher productivity in mass culture under high light (1500 µmol photons m^{-2} s^{-1} PAR) (Mitra and Melis, 2010; Polle *et al.*, 2003), while another mutagenesis effort failed to show higher productivities in mass culture of *Cyclotella* ssp. despite showing an improved photosynthetic efficiency(Huesemann *et al.*, 2009).

1.3.2 Challenges in regards to the genetic modification of algae.

While genetic manipulation clearly has potential in improving microalgae for biofuel production, there are also several problems associated with this strategy. Firstly the legal framework and public perception surrounding GM organisms is a challenge that any company growing GM organisms has to consider. The release of algae into the environment should be seen as unavoidable (Henley *et al.*, 2013), mainly because they are

microorganisms and no matter how well contained a system is, over realistic timescales (i.e. several years) GM algae will have the chance to escape into the environment. Therefore any large-scale culture of GM algae should be carefully considered on a case by case basis. The most comprehensive work on the potential of genetically modified algae to become established in the environment has been done by a group led by Prof. Kevin Flynn (University of Swansea). They have used extensive modelling of algal growth and predator-prey interactions to show that algae with high growth rates and high carbon content (either in lipids or polysaccharides) would be likely to become established and furthermore they would represent a food source of low nutritional value to zooplankton due to their suboptimal C:N:P ratios (Flynn *et al.*, 2012). Furthermore if the lipid profile is changed to produce drop-in fuels, this might make algae even more unpalatable because some of the compounds considered drop-in fuels are toxic to many forms of life (Flynn *et al.*, 2012; Radakovits *et al.*, 2010). So a scenario can be imagined where algae get released and dominate water bodies due to their high growth and low predation rates while also producing mildly toxic compounds that are good fuel precursors but not readily assimilated in the food chain.

On the other hand, GM for reduced light harvesting antenna length are much less likely to be stable even within algal bioreactors. This is because having a reduced-size light harvesting antenna is only beneficial to the cell in high light conditions where wild-type cells would experience photoinhibition (Melis, 2009). In low light conditions, where the rate of photosynthesis is not maximum, it is beneficial to have more chlorophyll in order to harvest more light. This means that algae experiencing natural variation in light levels would eventually adapt to express longer light harvesting antennae or they would show reduced fitness compared to their wild-type counterparts for most hours in the day (Flynn *et al.*, 2010).

It is also proposed that GM algae should be extremophiles so that their high growth rates are only achieved in specific, uncommon conditions thus minimising the probability of persistence or dominance upon release as well

as the potential for contamination in the growth apparatus (Radakovits *et al.*, 2010). Algae such as *Dunaliella salina*, an extreme halophile and *Pseudochorisystis ellipsoidea* an acidophile as well as thermophilic algae would under certain conditions satisfy this requirement. But case by case assessment will remain important in assessing the potential environmental impact of GM algae.

1.4 *Pseudochoricystis ellipsoidea*: a novel oleaginous microalga.

Satoh *et al.* (2010) report on the isolation of a fast-growing oleaginous green alga from a hot spring in Japan. This alga was named *Pseudochoricystis ellipsoidea* (Trebouxiophyceae) and its growth rates were reported as high as 3.46 g L^{-1} d^{-1} dry weight (DW) with a maximum biomass content of 7.2 g L^{-1}.

Further to its high growth rates, *P. ellipsoidea* is also an acidophile, showing maximum growth rates in pH 3-4 (Hiroaki Fukuda, personal communication). Optimal growth in low pH is highly desirable because it significantly reduces the contamination potential of industrial cultures as low pH inhibits the growth of most bacteria and other algae.

P. ellipsoidea accumulates triglycerides under nitrogen starvation with a chain length of C:16 to C:20 and a total lipid content of ≈ 30%. It also has the very unusual characteristic of accumulating 9.1% dry weight of linear hydrocarbons (C:17 and C:20) under N-starvation (Satoh *et al.*, 2010). These alkanes can be used directly as a liquid fuel. Hydrocarbon production is a characteristic that has only been reported in one other alga (*Botryococcus braunii*) albeit at longer chain lengths that cannot be directly used as transportation fuel (Banerjee *et al.*, 2002).

P. ellipsoidea possesses a very strong cell wall which is said to make cell disruption and lipid extraction particularly costly. Collaboration was developed between the DENSO Corporation and the University of Bath to

investigate this cell wall polymer. It was found that *P. ellipsoidea* possesses an algaenan cell wall (Smith-Baedorf, 2012). Algaenan is distinguished by its characteristic trilaminar structure (Atkinson *et al.*, 1972), resistance to hydrolysis by strong acids and bases as well as resistance to all organic solvents (Allard *et al.*, 1998; Blokker *et al.*, 2006) and known cell wall lytic enzymes (Atkinson *et al.*, 1972; Smith-Baedorf, 2012). Algaenan was also found to be resistant to most cell wall stains (Zych *et al.*, 2009).

1.5 Algaenan cell walls in microalgae.

Algaenan is a resistant biopolymer that is found in some species of green algae and in the genus *Nannochloropsis* (Eustigmatophyceae). This polymer is a very long chain heteropolyester whose chemical stability has made it particularly difficult to study and elucidate its structure and monomer composition. Due to their indigestibility, algaenan cell wall microfossils seem to be particularly abundant in some early Mesoproterozoic shales (approximately 1.6-1 billion years ago) (Dutta *et al.*, 2006; Sharma *et al.*, 2009). Algaenan cell walls have been mostly studied from a geochemical perspective as they seem to be important drivers of organic carbon export and important contributors to type I kerogens, thus giving rise to a significant proportion of fossil fuels consumed today (Derenne *et al.*, 1992; Gelin *et al.*, 1997; Versteegh and Blokker, 2004; Walters, 2006). Because of the presence of algaenan in some of the most important species considered today for biofuel production, the biological and industrial significance of algaenan cell walls becomes important but, so far, underappreciated. In the next few sections a review of the current knowledge on algaenan cell walls from a biological perspective and with focus on the green algae is attempted and important gaps in our knowledge identified.

1.5.1 Occurrence.

Algaenan occurs mainly in the division Chlorophyta, which encompasses the green algae and land plants. In this group algaenan has been found in two out of five classes; the Chlorophyceae and the Trebouxiophyceae (Kodner *et al.*, 2009). Within these two classes algaenan production shows no distinct evolutionary pattern with closely related species differing in their ability to produce algaenan containing cell walls (fig. 1.2.). Algaenan has also been found in the Eustigmatophyceae (Andersen *et al.*, 1998; Gelin *et al.*, 1997), a class of algae related to the golden (Chrysophyceae) and brown (Phaeophyceae) algae (Andersen *et al.*, 1998). In this class, algaenans have been described in the genus *Nannochloropsis* (Gelin *et al.*, 1997). Algaenan monomer composition is species or clade specific (Allard and Templier, 2001; Allard *et al.*, 2002; Berkaloff *et al.*, 1983; Blokker *et al.*, 2006, 1998b; Brennan *et al.*, 2012; de Leeuw *et al.*, 2006; Kodner *et al.*, 2009; Simpson *et al.*, 2003), thus the term algaenan does not describe a specific biopolymer but becomes a functional term for very long chain, aliphatic, non-hydrolysable, cell wall heteropolymers (Kodner *et al.*, 2009). The facts that algaenan is found in very distantly related groups of algae, resulting from primary (Chlorophyta) and secondary (Eustigmatophyceae) endosymbiosis (Keeling, 2010; Leliaert *et al.*, 2012) as well as the diversity these algaenans show in monomer composition (Versteegh and Blokker, 2004) seem to indicate that the ability to produce algaenan cell walls is subject to widespread convergent evolution. The phylogenetic pattern of algaenan occurrence, especially in the green algae where it is better studied, suggests that algaenan cell walls have been lost and re-evolved several times in this division. The possible reasons for this, as well as the ecophysiological implications of producing an algaenan cell wall are discussed later in this chapter. Finally, it is noteworthy that the verification of algaenan occurrence in a species currently requires a laborious cell wall purification (Allard and Templier, 2001; Allard *et al.*, 2002, 1998) and relatively few species have been properly screened (Versteegh and Blokker, 2004). Therefore algaenan could still be described in more classes of algae.

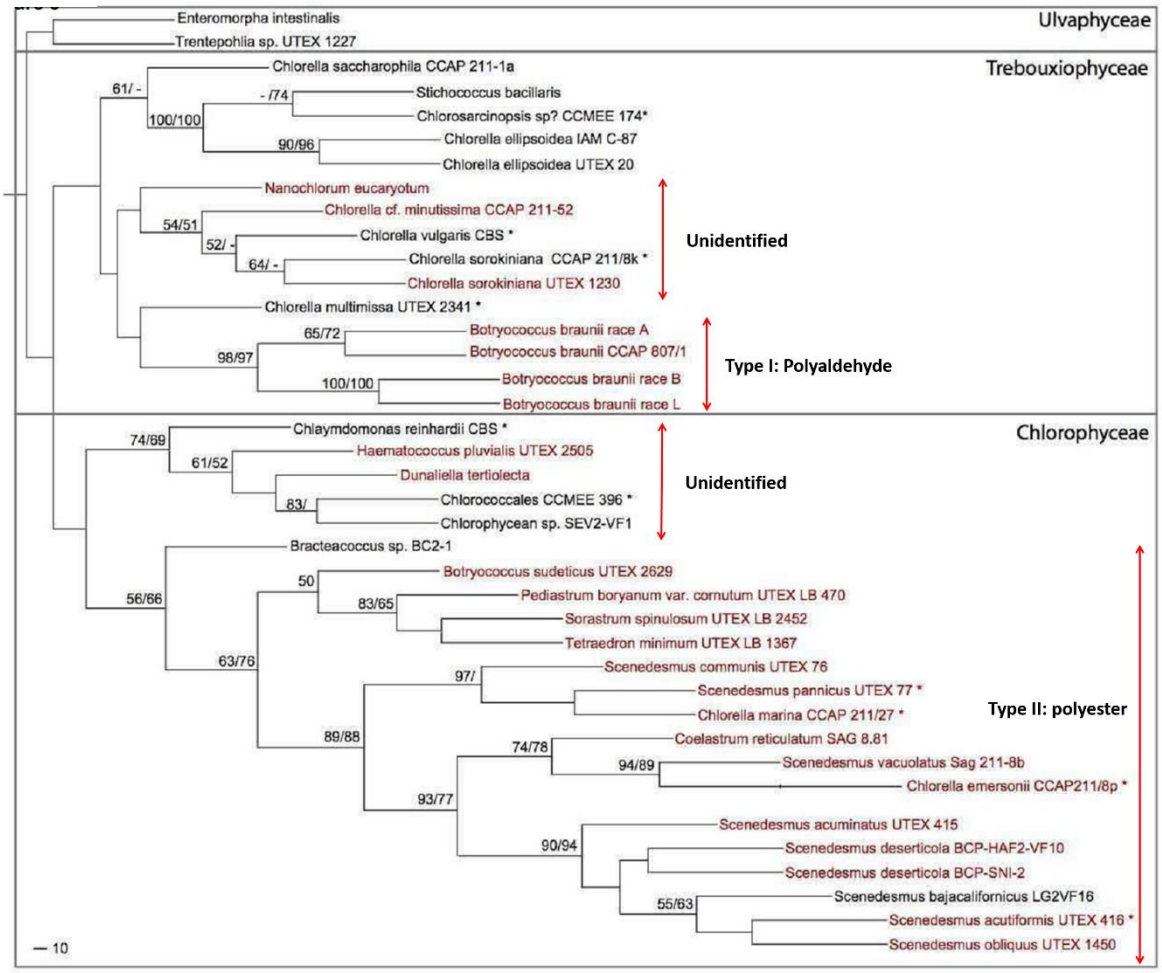

Figure 1.2. Consensus maximum like- lihood phylogeny of 18S SSU rDNA of Chlorophyta tested for algaenan. Taxa in red produce algaenan. Adapted from Kodner *et al.*, (2009)

1.5.2 Chemical composition.

Algaenan is an extremely resistant biopolymer both mechanically and chemically. It is resistant to common enzymes used to degrade cell walls (Atkinson *et al.*, 1972; Smith-Baedorf, 2012), it is also resistant to acid and base hydrolysis, acetolysis and non-oxidative chemical treatments (Allard *et al.*, 1998; Atkinson *et al.*, 1972; Versteegh and Blokker, 2004). The extreme insolubility of this biopolymer has greatly hampered efforts to elucidate its structure and composition as GC/MS and other mass spectrometry techniques as well as ^{1}H NMR and ^{13}C NMR could not be used to elucidate the structure and composition of algaenan. Furthermore, the harsh chemical treatments required to purify algaenan can introduce artefacts such as melanoidin-like polymers (Allard *et al.*, 1998) while different cell wall purification protocols

have yielded widely different results (Allard *et al.*, 2002; Atkinson *et al.*, 1972; Berkaloff *et al.*, 1983; Blokker *et al.*, 2006; Burczyk, 1987). It is, therefore, no surprise that 40 years after its discovery, the structure and composition of algaenan still remains ambiguous.

Algaenan was first discovered in *Chlorella fusca* (renamed *C. emersonii* since and likely to be renamed *Scenedesmus emersonii* in the future due to its phylogenetic position(Kodner *et al.*, 2009)), strain CCAP 211/8p (Atkinson *et al.*, 1972). When algaenan was initially described in green algae, it was misidentified as algal sporopollenin on the basis of being resistant to acetolysis and enzymatic hydrolysis. This, together with the fact that many algae produce secondary carotenoids (Burczyk, 1987), led to the assumption that algaenan is composed of ester- and ether-linked carotenoids and phenolic compounds (Good and Chapman, 1978). The application of solid-state ^{13}C NMR (Berkaloff *et al.*, 1983) showed that algaenan was an exclusively aliphatic polymer. Subsequent analyses have shown that known algaenans can be divided into three types: In the Eustigmatophyceae core algaenan consists mainly of very long chain (up to C_{36}) saturated alcohols, (α, ω)-diols, and mid-chain alcohols, linked by ether bonds (Gelin *et al.*, 1997). In *Botryococcus* spp. the core algaenan structure is a reticulated, unsaturated polyaldehyde (up to C_{32}), associated with very long chain hydrocarbons in race A (C_{40} average chain length) (Simpson *et al.*, 2003) and isoprenoid diols in race B (Metzger *et al.*, 2007), while in race L it is based on long isoprenoid chains (Derenne *et al.*, 1989), reflecting the fact that race L produces almost exclusively isoprenoid lipids. The third recognisable type of algaenans is found in the Chlorophyceae and the majority of studies have been carried out in this class. In studies of the species *C. emersonii, S. communis, Tetrahedron minimum* and *Pediastrum boryanum* it was shown that chlorophycean algaenan is a linear polyester of C_{16}-C_{36}, predominantly even-numbered, saturated fatty acids, ω-hydroxyacids, alcohols, α,ω-diols and dicarboxylic acids, possibly crosslinked by ether bridges (Allard and Templier, 2001; Allard *et al.*, 2002; Blokker *et al.*, 2006, 1998b)(fig. 1.3.).

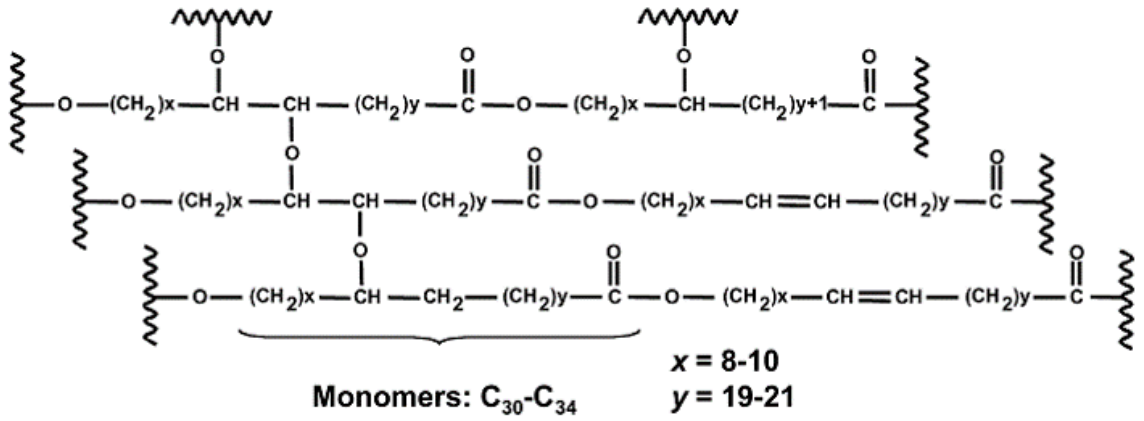

Figure 1.3. An illustrative simplified model of the algaenan present in the walls of the Chlorophytes *Tetraedron minimum* and *Scenedesmus communis* after Blokker *et al.* (1998). From Versteegh and Blokker (2004).

The relative percentages of these monomers are species-specific and may also be culture-specific as algae have been shown to alter their lipid profiles in response to different conditions (Olofsson *et al.*, 2012). These monomers are also found in the unbound neutral lipid profiles of the respective species(Blokker *et al.*, 1998b), however neutral lipid profile is not always a good indicator of algaenan occurrence and composition in a species(Allard and Templier, 2000). Low levels of aromatic compounds have been detected in *T.minimum* (Allard *et al.*, 2002) and some isoprenoid lipids have been detected in *Pediastrum braunii* algaenan (Blokker *et al.*, 2006). Some disagreement still exists over the degree of unsaturation of these monomers as well as the degree of ether crosslinking between polyester chains (Allard *et al.*, 2002; Blokker *et al.*, 1998b). It is also noteworthy that in all studies where complete degradation methods such as pyrolysis, TMAH thermochemolysis and RuO_4 oxidation methods were coupled with GC/MS to elucidate monomer chain lengths (Allard *et al.*, 2002; Blokker *et al.*, 2006), only 1% to 10% of products were GC-amenable (when mentioned). Allard *et al.* 2002 showed that high molecular weight products (400 – 2000 Da) were present with peaks corresponding to C_{20}-C_{30}, C_{50}-C_{60}, C_{80}-C_{90} and C_{110}-C_{120}

chains. Since C>100 chains are found in the kerogen fraction of fossil oil deposits it is hypothesised that they are also present in algaenan, however this result has not been indepentently verified. In the *Chlorophyceae*, algaenan has also been identified in the vegetative non-motile and red aplanospore stages of the commercially important alga *Haematococcus pluvialis* (Montsant *et al.*, 2001) but this algaenan has not been adequately characterised to our knowledge.

1.5.3 Biosynthesis.

Very little research has been done on the biosynthesis of algaenan and no algaenan-producer genomes had been publicly available until the sequencing of *Nannochloropsis gaditana* recently (Radakovits *et al.*, 2012). Therefore the evidence in this chapter comes from research on the biosynthesis of cutin, suberin and sporopollenin in higher plants and mainly *Arabidopsis thaliana* where it has been most widely studied. Algaenan can be considered an ancestral waxy polymer to the above and likely shares not only similar monomers but also part of the biosynthetic pathways. It is generally accepted that stearic (C16:0) and oleic (C18:1) acids synthesised in the chloroplast are the precursor molecules for all the above polymers (Ariizumi and Toriyama, 2011; Pollard *et al.*, 2008). These fatty acids are bound to CoenzymeA and are elongated and modified in the endoplasmic recticulum (ER) by anchored proteins (Cook and McMaster, 2002). Fatty acid elongase proteins determine the final length of the carbon chains in these monomers (Denic and Weissman, 2007). These lipids possibly in the form of fatty acyl-CoA or associated with other proteins are then secreted from the ER by lipophilic vessels to the extracellular space where they are deposited on the glucan primary cell wall and linked by ester bonds to the C-6 carbon of glucans (Rodriguez *et al.*, 1999) and polymerised in the form of extending plaques (Atkinson *et al.*, 1972)(fig. 1.4.) to form the secondary cell wall (Ariizumi and Toriyama, 2011; Pollard *et al.*, 2008).

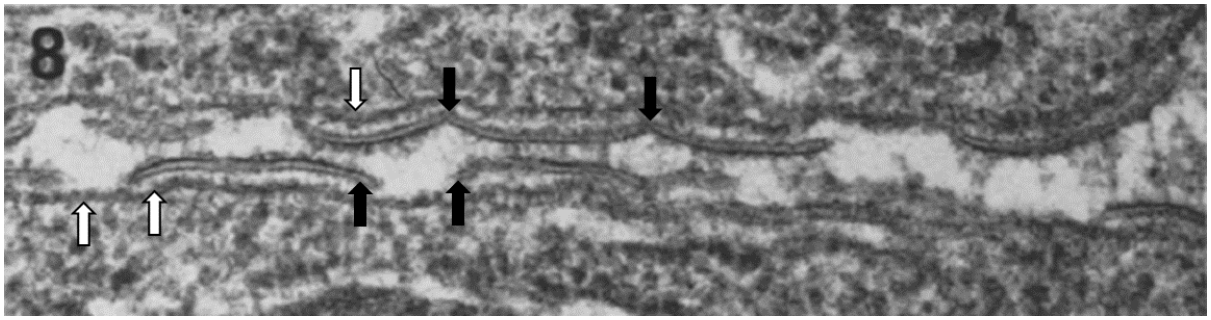

Figure 1.4. TEM image showing extension and fusion of algaenan plaques during cell division in *C. emersonii* (67000x magnification). Black arrows denote algaenan and white arrows denote plasma membrane. Adapted from Atkinson *et al.* (1972).

While several enzymes have been identified that are essential to sporopollenin synthesis such as FA-hydroxylases (Dobritsa *et al.*, 2009), FA-reductases (Ariizumi and Toriyama, 2011) and Acyl-CoA synthases (de Azevedo Souza *et al.*, 2009) as well as gene regulatory proteins (Jung *et al.*, 2006), the polymerisation step remains very poorly understood.

1.5.4 Ecophysiology.

Lipids are some of the most energy intensive metabolites produced by cells, hence their usage as a long-term energy storage in all Kingdoms of life, as well as the energy source that created and powered the current industrial age of humanity. Algaenan, being composed of very long carbon chains and little oxygen should then be one of the most metabolically costly cell walls possible. Producing an algaenan cell wall would certainly be more costly than cellulose which consists of ß,(1-4) glucose and thus requires little modification after photosynthesis. Furthermore, algaenan cell walls have to be shed at every division as they cannot be degraded by algal enzymes (or any other known enzymes) once they have been formed (Allard *et al.*, 2002; Atkinson *et al.*, 1972).

Algaenan isolated by Allard's improved method was 1-6% of biomass in most green algae and up to 11.4% in *Botryococcus* spp. (Allard *et al.*, 1998), 1.4% of biomass in *Nannochloropsis salina* (Gelin *et al.*, 1997) and 0.15% - 0.2% in *H.pluvialis* (Montsant *et al.*, 2001). When one considers that the cell, at each division, loses 1-6% of its dry mass and of a material that is

particularly costly to produce, it is logical to conclude that the manner in which these algae protect themselves is one of the most energy costly and wasteful ones found in nature. Indeed, wherever elsewhere waxy, organic polymers are found in nature, their function is long term protection. In plants they form cuticles in fruit (cutin) and epicuticular waxes in leaves, parts of the bark, protection of the roots (suberin) and the very resistant pollen exine wall (Albersheim *et al.*, 2011a). In microorganisms, similar polymers are found in resting stages of bacteria, algae and fungi (de Leeuw *et al.*, 2006). Therefore algaenan producers are unique in the fact that they produce and shed a waxy cell wall even when they are at exponential growth and divide every several hours to few days (Atkinson *et al.*, 1972; Rodolfi *et al.*, 2003).

Few hypotheses can be made on the reason some freshwater algae exhibit this peculiar pattern of cell wall production. The most obvious hypothesis would be that having an algaenan cell wall confers an advantage important enough to justify the energy expended for cell wall production. This advantage could be protection from dessication (Kodner *et al.*, 2009) and/or protection from infection/predation. Waxy polymers in plants are effective in preventing dessication and generally stated to be produced to prevent water loss and infection (Albersheim *et al.*, 2011b) although we are not aware of any experiments proving the efficacy of waxy cell wall polymers in preventing infection in plants or algae. Resistance to desiccation would be a beneficial feature for freshwater and terrestrial algae to resist seasonal drying of rivers and water holes as well as the ability to be transported between different water bodies by animals (Kodner *et al.*, 2009).

The desiccation resistance hypothesis would explain why algaenan is only found in freshwater and one shallow marine/brakish algae. However, there are no rigorous ecological studies on the distribution of algaenan producers in different habitats and desert algae lacking this polymer have been identified (Kodner *et al.*, 2009). The infection resistance hypothesis is based on the fact that no enzymes to digest algaenan have been found. Therefore algaenan producers would only be susceptible to predators that use

mechanical means of penetrating the cell wall such as rotifers (Lurling and Beekman, 2006) and chytrid fungi (Gutman *et al.*, 2009). However, there is no explicit evidence in favour of this theory and it is clear that there is a range of predators that can feed on algaenan producing microalgae so that infection/predation resistance is at best no more than a positive side-effect rather than an evolutionary driver.

1.5.5 Summary

Algaenan is a very chemically resistant biopolymer found in some green algae of freshwater origin and one genus of marine and brakish Eustigmatophyte. Because of its heteropolyester structure and extreme resistance to degradation it can be viewed as a naturally occurring bioplastic. Research has shown algaenan composition to be species-specific and also clade-specific, therefore the term algaenan encompasses algal cell wall polymers of aliphatic origin that are resistant to acetolysis. The chemical extraction proposed by Allard *et al.* (1998) is now accepted as the only proof of the presence of algaenan, however because algaenan has been defined on chemical rather than biological terms further research may still alter than definition. Considerable knowledge gaps on the biosynthesis and polymerisation of algaenan exist. Evidence from plant cuticles and sporopollenin in higher plants suggests that algaenan could be a relatively waterproof polymer and therefore conferring resistance to desiccation but this has not been explicitly tested. Due to its resistance to enzymatic attack, algaenan could also confer resistance to infection/predation from most algal parasites and predators with the notable exception of chytrid fungi and rotifers. These potential characteristics of algaenan cell walls are important in the field of applied phycology and biofuel production because of their potential effect on the culturing and downstream processing potential of certain algal species. Finally, the mechanical strength of this cell wall is of major importance to biofuel producers as cell disruption remains one of the most energy intensive and challenging steps in algal biofuel production.

1.6 Aims of this study.

The initial aim of this study was to improve the novel oleaginous green alga *P. ellipsoidea* for biofuel production by random mutagenesis and high throughput screening (Chapter 3) and to evaluate mutants in realistic growth conditions (Chapter 4). However as this study progressed it became apparent that a number of assumptions about the significance of algaenan had been made but never rigorously tested. In chapter 5 it was attempted to evaluate the significance of algaenan cell walls for the industrial culturing and processing of green algae by testing a screen of six species of fast-growing oleaginous microalgae that differ in their ability to produce algaenan. The focus was on designing experiments that would shed light both on the ecophysiological significance of algaenan and the suitability of algaenan producers to industrial culturing and downstream processing in the hope of assisting researchers in better species selection and matching species to purpose in the emerging field of applied phycology for biofuels.

2 Materials and Methods.

2.1 Materials.

2.1.1 Chemicals and lab consumables.

All nutrients and microelements required to make media and pH adjustments (as well as pH standards) for algal growth as well as DMSO and Nile Red for staining and chloroform/methanol for lipid extraction were purchased from Sigma-Aldrich. BODIPY$^{493/503}$ was purchased from Invitrogen. Cell-Tak, a protein coating to immobilise cells for microscopy, was purchased from BD Biosciences. Fertilisers to supplement ammonia and phosphate in DPM medium were purchased from BHGS. Glassware and pipette tips were supplied by VWR. All pipettes were serviced and re-calibrated at the beginning of this project.

2.1.2 Algal species.

Two strains of the novel microalga *Pseudochoricystis ellipsoidea* , named Ni and Obi, were provided by the DENSO Corporation. When *P. ellipsoidea* is mentioned without specifying the strain, the reader should assume strain Ni was used. The microalgal strains *Chlorella emersonii* CCAP 211/8p, *Chlorella vulgaris* CCAP 211/11b, *Chlorella vulgaris* (formerly named *C. minutissima*) CCAP 211/52, and *Chlorella zofingiensis* CCAP 211/51 were obtained from the Culture Collection of Algae and Protozoa and another newly isolated *Chlorella* sp., termed strain FC2-IITG (genebank accession number JX154075) was provided by Dr. Debasish Das (Indian Institute of Technology, Guwahati). For reasons of clarity and to avoid confusion strain *C. vulgaris* CCAP 211/52 will be referred to with its previous name (*C. minutissima*) hereafter.

2.1.3 Growth Media.

Two media were generally used for the culturing and maintenance of microalgae in this study. For most species and for long term maintenance in liquid or 1% agar, Bold's Basal Medium (BBM) (Bischoff and Bold, 1963) was used. This contained 2.94 mM $NaNO_3$, 430.6 µM K_2HPO_4, 1.29 mM KH_2PO_4, 427.8 µM NaCl, 304.3 µM $MgSO_4 \cdot 7H_2O$, 170.1 µM $CaCl_2 \cdot 2H_2O$, 184.7 µM H_3BO_3, 46.9 µM H_2SO_4, 44.8 µM $FeSO_4 \cdot 7H_2O$, 134.3 µM Na_2EDTA, 54.6 µM $ZnSO_4$, 11.4 µM $MnCl_2$, 4.93 µM MoO_3, 9.84 µM $CuSO_4$, and 2.67 µM $Co(NO_3)_2$. A four-fold dilution of BBM medium (BBM/4) served as the standard medium in chapter 5. This dilution took place to decrease the growth period required to reach stationary stage (see chapter 5)

For the maintenance and culturing of the six species screen in chapter 5 species were maintained in a four-fold dilution of BBM medium (BBM/4). This was also the standard medium used for 6 spp. screen experiments (chapter 5) unless otherwise stated.

For growth of *P. ellipsoidea* in raceway ponds a minimal media developed by DENSO for the large scale culturing of *P. ellipsoidea* (Hiroaki Fukuda, personal communication) was used. This media was termed DENSO Pond Media (DPM) and it contained; 186.70 µM $(NH_4)_2SO_4$ (Sulphate of ammonia fertiliser, 21-0-0, BHGS), 15.20 µM KCl, 7.03 µM $MgSO_4 \cdot 7H_2O$, 12.25 µM $Ca(H_2PO_4)_2$ (superphosphate fertiliser, 0-17-0, BHGS), 478.91 µM FeEDTA, 754.77 nM H_3BO_3, 360.87 nM $MnSO_4 \cdot 7H_2O$, 695.46 nM $ZnSO_4 \cdot 7H_2O$, 801.00 nM $CuSO_4 \cdot 5H_2O$, 9.71 nM Na_2MoO_4 and 359.44 nM $CoCl_2$. Fertiliser stocks were centrifuged to remove particulates before inoculation. For growth of seed cultures and in photobioreactors, a fourfold concentrated version of the DPM media was used (4DPM).

All media and glassware used for bench-scale culturing and culture maintenance were sterilised by autoclaving at 121 °C for 15 minutes. pH was adjusted to 6.5 for BBM and 4.0 for DPM by addition of 1 M HCl/NaOH and measured by a pH electrode (Fisher Scientific) that was calibrated with

standards of pH 4, pH 7 and pH 10 depending on the range measured. Photosynthetically active radiation (PAR) was measured using a LiCor 250A light meter. Chemicals were weighed using a Sartorius analytic A200S mass balance with a sensitivity of 0.0001 g.

2.1.4 Growth room setup.

A temperature controlled Fitotron plant growth room (WEISS Gallenkamp) at Bath University was converted to an algal growth room to provide space for growing algal cultures for the different experiment described below and to house the vertical photobioreactor units. One side of the growth room consisted of a row of white fluorescent lights and tungsten tubes mounted to the ceiling and a shelving unit of two shelves spanning the length of the lights installation. Light levels were approximately 250 µmol photons m^{-2} s^{-1} PAR on the top shelf, 100 µmol photons m^{-2} s^{-1} PAR on the bottom shelf and 70 µmol photons m^{-2} s^{-1} PAR on the floor. Magnetic stirrers were housed on the shelves and rotary shakers on the floor of the room. The other side of the room was reserved for the gas delivery equipment and the vPBRs as described below. Algal cultures could be bubbled (if desired) with 2.5% CO_2. This was provided by an oil-free air pump and a 100% CO_2 cylinder (BOC) that were mixed by a GM-T Gas Mixer (BSL Gas Technologies). The gas mixer was adjusted to 2.5% CO_2 measured by a CO_2 sensor (Crowcon). Both gases were filtered by a 0.01 µm coalescing filter and an activated carbon filter (Bosch) as well as a 0.2 µm hydrophobic sterile filter (Fisher). Gas bubbling was done by sintered glass spargers with 16 µm – 40 µm pore size (DasGip, Germany). The growth room was set to a nominal temperature of 22 °C. At this temperature setting the culture temperature was found to be 25 °C which was the desired temperature for algal growth. The light:dark cycle was set to 16:8 hours with lights coming on at 06:00 and turning off at 22:00 every day. A Crowcon Cellarsafe CO_2 monitor with a remote alarm was placed in the growth room to monitor CO_2 levels.

2.1.5 Bubble column photobioreactor (PBR) setup.

14 vertical, bubble column photobioreactors with a working capacity of 8 L each were purchased from ExAlga (UK) and set up in the algal growth room. These consisted of transparent acrylic tubes of 10.7 cm diameter and 102 cm length with a conical bottom that featured one small opening for CO_2 delivery approximately 2 cm off the bottom, and a larger opening at the bottom of the cone for sampling and harvesting. The top of the tubes was capped with foam bungs. Mixing and CO_2 were provided by bubbling a gas mixture of 2.5% CO_2 in air at the rate of 1.2 sL min^{-1} through a yellow (200 µl) pipette tip (fig. 2.1.). Light levels were approximately 80 µmol photons m^{-2} s^{-1} PAR at the light facing side of the tubes. The tubes and media were sterilised before inoculation by bubbling with ozone for 60 mins. After harvesting the tubes were cleaned with de-ionised water (dH_2O) and 70% ethanol.

Figure 2.1. 8 L bubble column photobioreactor units at the University of Bath (main image) and detail of the gas delivery via a pipette tip (white arrow) and sampling port (black arrow) (inset).

2.1.6 Raceway pond setup.

Two raceway ponds of 500 L nominal capacity were set up in a glasshouse at the University of Bath. The purpose of this was to assess the growth and productivity of wild-type *P. ellipsoidea* in realistic conditions and to, eventually, compare it to the productivities of any mutants obtained from

the mutagenesis procedure described in chapter 3. Two raceway ponds were constructed by Designer Composites Ltd. (UK) using designs provided by Hiroaki Fukuda (DENSO) as a guide. The ponds were made of fiberglass and coated with a polyester-based, food-grade resin. The dimensions were 315 cm x 165 cm x 50 cm (LxWxD). The paddle wheels were made of transparent polycarbonate and were designed and constructed by a team led by Dr. Paul Frith (Department of Mechanical Engineering, University of Bath). An electric motor (RS) drove the paddlewheel in order to provide mixing for the ponds, at a speed of 3.5 rpm. Pure CO_2 (BOC) was delivered at a rate of 0.15 sL min^{-1}, by a 16-40 μm pore size sintered glass sparger (DasGip) located upstream from the paddlewheel. The gas stream was filtered through a 0.2 μm hydrophobic sterile filter (Fisher). Two high-pressure sodium (HPS) lamps were located above each pond. These were automatically switched on when environmental light levels were very low (i.e. dawn and dusk) in order to give algae grown in the ponds a similar photoperiod as the algae grown in PBRs (16:8 h light:dark). The lamps delivered 80 μmol photons m^{-2} s^{-1} PAR to the surface of the ponds.

2.1.7 Microscopy.

Haemocytometer images for cell counts were taken using a Nikon eclipse TE2000-5 inverted microscope under 20x magnification.

A Nikon 90i Eclipse confocal microscope was used to take high quality images at 100x magnification under brightfield or confocal mode. A Nikon Digital Sight DS-U1 colour camera was used for brightfield images of cells and communities in wastewater and anaerobic digestate. In the confocal mode a D-Eclipse C1 camera was used for lipid detection. A 488 nm laser was used for the excitation of chlorophyll and $BODIPY^{493/503}$ with a 570LP and a 530/30 detector used to detect fluorescence of chlorophyll and BODIPY respectively. For Nile Red, a 543 nm excitation with a 570LP detector were used.

2.1.8 Software.

The Microsoft Office suite was used for much of the work in this thesis. Microsoft Word was used to write this thesis, Microsoft Powerpoint was used to produce diagrams and label images and Microsoft Excel was used for the majority of graphs, to calculate standard errors and error bars as well as t-tests and correlation analysis. Minitab 15 was used for most of the statistical analyses such as normality tests, significance tests with non-normal data, ANOVA and regression analyses. Townend (2006) was used as a guide for statistics and data analysis.

Nikon NIS Elements BR was used to capture light microscopy pictures and Nikon EZ-C1 was used to capture and process confocal images. ImageJ 1.48 was used to process all microscopy images and perform cell counts, add scale bars and measure cell wall thickness in TEM images.

2.2 Methods.

2.2.1 Culturing overview.

2.2.1.1 Long-term culture maintenance.

Culture maintenance was performed in the following way; all species and strains were kept on 1% agar – BBM plates in a small incubator (Sanyo MCO-15AC) at 15 °C supplemented with 3-5 µmol photons m^{-2} s^{-1} PAR of light from a white fluorescent tube. Species kept in that way were transferred onto fresh agar plates every three to four months to ensure viability. Frequently used species were also maintained in liquid stocks. These consisted of 100 ml cultures in 250 ml conical flasks with BBM and were kept in a Sanyo MLR-351 "environmental test chamber" at 25 °C, shaken at 120 rpm by rotary shakers (Sanyo MIR-S100) and supplemented with 150 (±15) µmol photons m^{-2} s^{-1} PAR in a 16:8 hour light:dark cycle. These were subcultured every 7-21

days to keep them actively growing and provide biomass to seed larger culture vessels as described below.

2.2.1.2 Culturing P. ellipsoidea for mutagenesis.

P. ellipsoidea was cultured for mutagenesis in a Sanyo environmental test chamber, shaken at 120 rpm and under 150 µmol photons m^{-2} s^{-1} PAR. 100 ml of BBM cultures in 250 ml conical flasks would be inoculated with $0.05*10^6$ cells ml^{-1} of *P. ellipsoidea* and grown to a density of $1\text{-}10*10^6$ cells ml^{-1} before harvesting for mutagenesis.

2.2.1.3 Maintenance of a mutant library

Mutant strains were maintained in 96-well microplates with a working volume of 200 µl. These were kept in an incubator (Sanyo MCO-15AC) at a low light intensity (3 µmol photons m^{-2} s^{-1} PAR) and temperature (15 °C) in order to significantly reduce growth rates for long-term maintenance. Mutants of particular interest were also kept as colonies on agar plates.

2.2.1.4 Culturing P.ellispoidea for large scale assessment; master cultures to pond cultures.

A number of steps had to be taken to grow enough biomass to seed tubes and ponds for large-scale assessment of *P. ellipsoidea* productivity. Generally the master cultures were 100 ml conical flasks. If there was any doubt about the sterility of those, they would be re-started from agar-grown colonies. For PBR or raceway pond experiments, 90 ml of master culture would be inoculated in 1 L of 4DPM and grown for 7-10 days, stirred by a magnetic stirrer (250 rpm) at 100 µmol photons m^{-2} s^{-1} PAR. This culture would then be used to seed the 8 L PBRs to an initial concentration of $0.5\text{-}1*10^6$ cells ml^{-1}. These would be grown for 7-10 days to provide inoculum for

the raceway ponds or for 14-21 days to monitor *P. ellipsoidea* productivity and lipid accumulation. One 8 L PBR culture would be used as inoculum in each pond giving an initial cell concentration of 0.1-0.5*10^6 cells ml^{-1}. The content of two PBRs were mixed in a 20 L Jerry can before inoculation to ensure that the two ponds had exactly the same cell numbers at the onset of growth curves. For comparing productivity of *P. ellipsoidea* in raceway ponds and PBRs two ponds and three PBRs were set up as described above. Growth monitoring was performed in three different ways; cell counts by flow cytometry, optical density at 550nm and dry weight (DW) measurements as described below.

Pond media was made using water directly from the mains supply. A single jet ¾ inch water meter with 0.1 L accuracy (RS) was attached to the hose pipe and was used to measure 500 L of water in the ponds. This was left mixing gently overnight to equilibrate with temperatures in the glasshouse. Stock solutions of nutrients and microelements were prepared at 1000x concentration and added to the ponds while gently mixed. Then two 100 ml samples were taken and their pH was adjusted in the lab to pH 4. From this we extrapolated on how much concentrated HCl (10.15 M) would be needed to adjust the pH in the ponds. Algae were generally inoculated in the morning in order to give them as large as possible a photoperiod to adapt to the new environment. After harvesting the ponds and paddlewheels were cleaned by hand using 1% bleach and tap water. *P. ellipsoidea* was inoculated at an initial concentration of 0.7*10^6 cells ml^{-1} and grown in the ponds for 20 days.

2.2.1.5 Growth in autoclaved and non-autoclaved anaerobic digestate and wastewater.

For growth in sterile and non-sterile anaerobic digestate (AD) we obtained anaerobic digestate from Andigestion Ltd. The anaerobic digester was located in Hollsworthy, UK and digested a mixture of abattoir waste supplemented with agricultural waste. AD was filtered through a 100 µm

nylon mesh filter to remove coarse particulates. The crude AD contained approximately 5 g L^{-1} NH_4-N and 0.1 g L^{-1} PO_4-P. To create an optimal medium AD medium for our six-species screen we grew the algae in round-bottom, 96-well plates (Corning), initially at three different dilutions in dH_2O (0.2 %, 0.4 % and 1% vol/vol) and subsequently in 0.4 % AD with and without the supplementation of vitamins (1.2 mg L^{-1} thiaminhydrochloride and 10 µm L^{-1} cyanocobalamin, sterile filtered through a 0.2 µm syringe filter (Millipore)). We found that all six species did not require vitamin supplementation for growth and henceforth a simple dilution of the filtered AD was used as the AD medium (ADM) (0.4 % vol/vol final concentration).

For growth in sterile and non-sterile wastewater (WW), we obtained final wastewater effluent from a municipal wastewater treatment plant in Saltford, UK. Nutrient removal in the plant was performed by iron dosing of primary settled WW followed by trickling filters. WW was kept in a cold room in the dark for ~14 days before the onset of experiments.

In both cases the "sterile" treatment was created by autoclaving the medium. All species were adapted to the sterile ADM and WW respectively for 14 days before the onset of experiments. On day0 the six species were inoculated in triplicate in 100 ml conical flasks with 50 ml of media. All species were diluted as necessary so that all flasks had equal OD_{550}. Two rotary shakers (one per treatment) were placed in a growth room under fluorescent lights in such a manner to minimise differences in light intensity across the growing surface of the algae. On the brighter side of the shakers the intensity was 199 ± 1 µmol photons m^{-2} s^{-1} PAR and on the darker side it was 182.5 ± 0.5 µmol photons m^{-2} s^{-1} PAR. The six species were placed on the shakers in such a manner so that the sterile and non-sterile treatment for a certain species always received the same light intensity and the respective positions on the shakers were changed daily to minimise the difference in the light received across the whole growing period between different species.

2.2.1.6 Growth for ultrasonication and TEM.

For ultrasonication experiments and TEM, 50 ml of algae were grown in 100 ml conical flasks (n=3) on a rotary shaker, shaken at 120 rpm and irradiated with 190 (± 10) µmol photons m^{-2} s^{-1} PAR. The flasks were rotated every two days to minimise differences in light levels. Cell growth was monitored with a Guava EasyCyte flow cytometer (Merck Millipore). Before flow cytometry, 1 ml of the algal cultures in eppendorf tubes were sonicated in a sonic bath for 15 min to break up cell aggregates that occur in the exponential growth stage of some of those species. This gentle sonication method was found to effectively break cell aggregates and increase the accuracy of cell counts without reducing the viability of those cells.

2.2.1.7 Growth for lipid extraction, biomass analysis and hydrothermal liquefaction.

For lipid extraction, hydrothermal liquefaction, biochemical and elemental analysis, algae were grown in 8 L vertical photobioreactors (n=1) irradiated with 80 µmol photons m^{-2} s^{-1} PAR on the light-facing side of the reactors and a light path of 10.6 cm (tube diameter). Mixing and CO_2 were provided by bubbling with 2.5% CO_2 at a rate of 1.2 L min^{-1} at the bottom of the vPBRs. Growth was monitored by optical density at 550 nm and the algae were harvested when all six species had reached stationary stage (21 days). Biomass productivity was assessed by lyophilising 3 ml of culture in triplicate for 24 hours and weighing.

2.2.2 Sonication for de-aggregation of cells.

A Fisher Sonic water bath (FB15046) with 30 W power and 50/60 Hz frequency was tested as a means to break cell aggregates of *P. ellipsoidea* at early exponential stage or whenever the cultures were deemed too clumped to get accurate cell counts. 1.5 ml microcentrifuge tubes containing 1 ml of algal

culture were floated in the sonic bath using a foam tube rack so that the full volume of the culture was submerger in the bath. For the initial trial 10 µl of sample were removed every 5 min and counted using a flow cytometer and the cell number were compared to the control (no sonication). This was done up to 50 min. 20 minutes of sonication was found to be adequate for breaking cell aggregates for the purposes of mutagenesis and cell counts and this was the time used in subsequent experiments.

2.2.3 Growth monitoring.

All samples were taken in the morning (09:00-11:00) and kept at 4 °C if the samples could not be measured immediately. Cultures in early exponential stage were gently sonicated for 20 mins to break cell aggregates. Each sample was measured twice and an average was obtained.

Cell division rates were calculated using the equation:

$$Div\ Day^{-1} = \frac{\ln(\frac{N_1}{N_0})}{\frac{t_1 - t_0}{\ln(2)}}$$

where N_1 and N_0 are the cell densities at end and beginning of cell growth respectively, and t_1 and t_0 are the time points at which exponential growth stopped and started, respectively.

2.2.3.1 Cell counts by haemocytometer.

An impoved Neubauer Haemocytometer was used to initially measure the cell density of the growing cultures. Cell counts were performed in such a way that at least 100 but no more than 300 cells were counted on each sample while counting from at least four of the main squares or five of the central subdivision squares. If the samples were too concentrated for accurate counts, they would be diluted appropriately.

2.2.3.2 *Flow cytometry.*

Cell counts were performed using a Guava EasyCyte 6-2C flow cytometer (Merck Millipore). This uses a 488 nm (blue) laser to read single cells as they pass through a capillary. Forward scatter area (FSC-A) was used as a proxy for cell size and chlorophyll fluorescence in the red channel (665-715 nm) was used as an indicator of viability. Specifically, when settting up the flow cytometer, glass beads and bleach-killed cells were used to set the zero value for red fluorescence and any cells showing a positive red fluorescence value were counted as viable. Voltages in the flow cytometer were set at 13.5 for FSC and 2 for RED.

It is important to note that, while flow cytometers are generally not considered to be accurate in cell counting due to their variable flow rates, the Guava Easycyte measures the volume of sample flowing through the capillary and calculates precise cell counts. Flow cytometry also has the advantage of less time required per sample (seconds rather than several minutes) and more individual cells counted (1000-5000 against 50-200 cells in haemocytometer counts).

Samples were diluted in 1 ml Eppendorf tubes as necessary to obtain a cell density of $<0.5*10^6$ cells ml^{-1} as above this density the flow cytometer tends to underestimate cell numbers.

2.2.3.3 *Optical Density.*

Optical density (OD) was measured by adding 1 ml of culture to a cuvette and recording OD at 550 nm using a Merck Spectroquant Pharo 300 (Merck Millipore). A wavelength of 550 nm (green) was used as chlorophyll shows minimum absorption of light at this area of the spectrum and thus the measurements taken would be proportional to biomass rather than chlorophyll content. An average of two measurements was used for each sample and ddH_2O was used in all cases as a blank (OD=0) as this had the same absorption as sterile BBM and DPM. Samples with an OD $\geq$ 0.7 were

diluted appropriately and OD values calculated by multiplying by the dilution factor.

2.2.3.4 Dry weight.

To determine the dry weight (DW) of biomass 1.5 ml of culture was added to pre-weighed eppendorf tubes and centrifuged at 12000 rpm for 5 minutes in an Eppendorf 5415C centrifuge. The supernatant was removed and another 1.5 ml of culture was added to the tube and centrifuged and the supernatant removed giving a total sample volume of 3 ml. The tubes with pellets were stored at -80 °C and freeze-dried at -40 °C for 24h using a Savant Speed Vac Concentrator connected to an Edwards Modulyo FreezeDryer with an Edwards EMF10 oil mist filter. After freeze drying the tubes were re-weighed to determine the amount of dry biomass present in each tube and the dry weight in grams per litre (g L^{-1}) was calculated. Weighing was performed using a Sartorius analytic A200S mass balance with sensitivity of 0.0001 g.

2.2.4 Harvesting and dewatering of raceway ponds.

Harvesting was done through a sump at the bottom of each pond. At the end of each growth period an I/P 77410-10 peristaltic pump with an I/P standard pumphead (ColePalmer, UK) was used to gently pump the algae into a 1000 L capacity settling tank (Smiths of the Forest of Dean, UK) with a conical bottom.

2.2.4.1 Flocculation

Flocculation was chosen as the main dewatering step. As discussed in the introduction, dewatering is one of the main problems with the viability of algal biofuels at present. Flocculation was chosen as the main dewatering step in this system because it is a low capital investment method requiring only a settling tank and a chemical flocculant. Autoflocculation did occur in *P.*

ellipsoidea but at a very slow rate (approximately 48 h) and cells were very easily resuspended making autoflocculation an unreliable method for this species. The work of choosing a suitable chemical flocculant and optimising flocculant concentration and flocculation pH was undertaken by Jane Lapworth as part of an undergraduate thesis (Lapworth, 2012) and is not described here in detail. Briefly, chitosan (practical grade, from shrimp shells, Sigma-Aldrich) in a concentration of 20 mg L^{-1} and pH of 10-11 was determined to be the best flocculant. Chitosan stock was made by dissolving 5 g L^{-1} of chitosan in 5% glacial acetic acid. This was added to the ponds or tubes at an appropriate amount to achieve 20 mg L^{-1} final concentration and stirred gently for 30 minutes either by the pond paddle wheel or gentle bubbling in the PBRs. Two 100 ml samples were then taken and gently stirred by a magnetic stirrer while 1 M NaOH was slowly added until flocculation occurred. The exact pH at which flocculation looked best (visual assessment) varied somewhat from day to day but was always between pH 10 and pH 11. We then extrapolated to the amount of NaOH needed to adjust the pH of the ponds or PBRs and 10 M of NaOH was added in appropriate amounts while gently mixing. Eventually mixing was ceased and the algae were allowed to settle. Flocculated material was then harvested from the bottom of the PBRs or the bottom of the harvesting tank for raceway ponds. The harvested biomass was further settled overnight in 20 L Jerry cans and the supernatant was removed by gentle pumping.

2.2.4.2 Centrifugation

Further dewatering of the biomass was performed by centrifugation using a Beckman Coulter Avanti J-25 centrifuge with a JLA – 10.500 rotor. 500 ml vials were centrifuged at 5000 rpm for 5 mins at room temperature. The algal paste was then removed from the vials and spread on 140 mm diameter petri dishes which were frozen at -80 °C.

2.2.4.3 Freeze-drying

When biomass was needed for downstream experiments such as lipid extraction the petri dishes containing the algal biomass were sealed with Parafilm in which several small holes were made using a needle or forceps. These were lyophilised in an Edwards Modulyo Freeze-Dryer with an Edwards EMF10 oil mist filter in a vacuum bell jar for 48-72 h.

2.2.5 UV Mutagenesis.

The UV mutagenesis protocol was adapted from Smith-Baedorf (2012). 25 ml of *P. ellipsoidea* at early exponential growth (10^6 cells ml^{-1}) was added to a petri dish and placed directly under a 15W UV lamp (≈53cm distance). Samples were taken at 0, 5, 10, 15, 18, 20, 22, 24, 26, 28 and 30 minutes and diluted by 1 in 500. 30 µl of the diluted samples were then aseptically plated on to plates (n=3) with BBM agar 1% supplemented with 0.1% sodium acetate as a carbon source. The plates were stored in a growth room at 25° C, initially for 24 hours in the dark to prevent photolyase and photo-inducible DNA repair mechanism activity (Sinha and Häder, 2002). After 24 h of darkness, plates were stored for 21 days under 80-100 µmol photons m^{-2} s^{-1} and a 18:6h light:dark cycle. When algal colonies were clearly visible on the plates, counts were performed to determine the UV exposure that led to 90% mortality. Subsequent mutagenesis trials were performed in the same manner as previously but only sampling at the time point of 90% mortality.

2.2.6 EMS Mutagenesis.

EMS mutagenesis was initially based on a protocol provided by Saul Purton (University College London) and described in Smith-Baedorf (2012). This protocol involved centrifugation and re-suspension of cells in phosphate buffer before treatment with EMS but it was found that *P. ellipsoidea* do not pellet well in the early exponential growth and thus replicates for determination of the right exposure to EMS were not possible (data not

shown). The protocol was modified as follows: 90 µl of EMS (Sigma-Aldrich) were added to 5 ml (1.8% w/v) of early exponential phase *P. ellipsoidea* (10^6 cells ml^{-1}), vortexed and incubated in an orbital shaker in the dark (reducing photo-inducible DNA repair mechanisms). 0.5 ml samples were taken at regular intervals from 0 minutes exposure (control) up to 5 hours exposure and mutagenesis was quenched by adding 0.5 ml of sterile filtered 5% w/v sodium thiosulphate and vortexed for 30 s. After inactivation, samples were immediately diluted by a factor of 1:250 and 30 µl aliquots of these samples were plated on BBM 1% agar plates (n=3) supplemented with 0.1% w/v sodium acetate as a carbon source. Plates were stored in the dark for 24 h and then in a growth room at 25° C under 80-100 µmol photons m^{-2} s^{-1} and 18:6 h light:dark cycle for 21 days. Colony counts were performed to determine the exposure necessary to achieve 90% mortality.

2.2.7 FACS screening.

A Becton Dickinson FACS Vantage SE, located at the University of Bristol was used to screen and sort mutant and wild-type populations. Mutagenised cultures were screened for low chlorophyll cells (putative tla mutants) and calcofluor fluorescent cells (putative cell wall mutants) excited with a 488 nm and 355 nm laser and emission detection at 667/30 nm and 440/40 nm respectively for chlorophyll and calcofluor. Negative controls were set by unstained wild-type cells. Sorted cells were inoculated on agar plates for maintenance and assessment of clonal cultures and liquid cultures for re-sorting to improve characteristics or reduce false positives.

2.2.8 Screening of mutants: outline.

After mutagenesis, a small proportion of the culture was diluted appropriately and spread on agar plates. 20 to 40 agar plates were made after each mutagenesis aiming at a calculated 1000 to 2500 colonies each time. The rest of the mutagenised culture was transferred to 100 ml conical flasks and

cultured in the growth chamber to be used for FACS screening. Colonies on agar plates after 21 days of incubation were visually screened for colour mutants (pale green/yellow) and unusually shaped colonies. Any such colonies were transferred to 96-microwell plates for long term maintenance and further screening (see section 2.2.1.). Liquid cultures, along with replicate wild-type cultures were screened by FACS for low chlorophyll autofluorescence (putative tla mutants) and the presence of Calcofluor fluorescence (putative cell wall deficient mutants).

2.2.9 Staining for microscopy and flow cytometry.

Nile Red (9-diethylamino-5-benzo[α]phenoxazinone) (Sigma-Aldrich) and BODIPY493/503 (4,4-difluoro-1,3,5,7,8-pentamethyl-4-bora-3a,4a-diaza-s-indacene) (Invitrogen) were used to visualise lipids. 24 µl of DMSO were added to 100 µl of culture to aid the penetration of stains through the cell wall and mixed. 1.5 µl of Nile Red or 0.2 µl of BODIPY were added and mixed before the total volume was brought to 1 ml by adding distilled water (dH2O) (Satoh *et al.*, 2010). Samples were kept in the dark and fluorescence was measured 10 to 30 minutes after staining.

For the isolation of mutants using FACS a vital staining method is extremely important. For cell wall staining cells were inoculated with 0.01% w/v Calcofluor white (7-(diethylamino)-4-methylchromen-2-one) for 10 minutes in the dark. This method has been shown to not significantly affect the viability of cells (Smith-Baedorf, 2012).

2.2.10 Semi-quantitative staining for comparison of lipid accumulation.

As the main focus of this study is the suitability of algae for biofuel production, a rapid and reliable method for the quantification of lipids in algal cells was highly desirable in order to facilitate selection and screening of mutants as well as for monitoring the effect of different growth systems and

conditions on lipid productivity. A microwave-assisted, flow cytometric method for the quantification of lipids based on work by Chen *et al.* (2010) using the BODIPY$^{493/503}$ (4,4-difluoro-1,3,5,7,8-pentamethyl-4-bora-3a,4a-diaza-s-indacene) (Invitrogen) neutral lipid vital stain was developed by Raleigh Jenner-Hillard (Jenner-Hillard, 2013) under the author's guidance as part of an undergraduate research project. The method development is not described in detail here but the final method for lipid quantification was as follows; a few millilitres of algal sample were counted and diluted appropriately for flow cytometry, then 10 µl of culture were transferred to a glass microwave vial containing 100 µl of dimethylsulfoxide (DMSO). The sample and DMSO mixture was then heated in a Monowave 300 laboratory-grade microwave coupled with a MAS 24 autosampler (Anton Paar) to 60 °C with stirring at 600 rpm for zero seconds and allowed to cool. Heating to 60 °C for t=0s effectively means that the microwave brings the sample up to the desired temperature but as soon as that temperature is reached the sample is immediately cooled down. Once cooled down, 880 µl of ddH_2O and 10 µl of 1 mM BODIPY$^{493/503}$ in DMSO were added to the vial and vortexed immediately. The vials were incubated at room temperature in the dark for 10 mins before being transferred to an Eppendorf tube for flow cytometry. The above process was done in triplicate for each sample and measured twice in the flow cytometer. Typically 1000 viable cells were measured in each run. BODIPY$^{493/503}$ fluorescence was measured in the Green detector (525 ± 15 nm). Voltage for the green channel was set to 2.18. The value of the area of green fluorescence (GRN-A) was used as a relative proxy for lipid content.

2.2.11 Ultrasonication for cell disruption.

A number of different methods for cell disruption were assessed during this project in order to develop a replicable method for comparing the mechanical cell wall strength of different species of algae. These included bead beating, nitrogen decompression, French cell press, homogenisation and ultrasonication. The only two methods that reliably fractured algal cells were

French cell press and ultrasonication. The later method was used to assess mechanical cell wall strength. 8 ml of algal cultures were centrifuged and re-suspended in a sonication buffer containing 50 mM Tris-HCl buffer (pH 7.5), 200 mM NaCl, 1 mM EDTA and 1 % (w/v) SDS. Ultrasonication was carried out in 15 ml Falcon tubes in ice and water, using an exponential probe half-way submerged into the culture to avoid foaming. The sonicator used was an old MSE sonicator (serial PG1341, code 4/77 MK2), fitted with a new "exponential" probe. The amplitude used was 21 μm. Algal cultures were ultrasonicated for a total of 15 minutes in 1 minute bursts with 4 minutes rest in ice. The probe was buffed using 1200 grade emery paper between each species to prevent loss of efficiency. Cell disruption was monitored by adding 0.4% Trypan Blue to the algal cultures and manually counting the undisrupted cells from light microscopy pictures using the ImageJ software. Algal cells were imaged slightly "out of focus" to better image the cytoplasm and cells that were able to exclude the stain were counted as undisrupted. This was necessary because cells with rigid walls can sometimes retain their shape even when walls and membranes have been significantly disrupted.

2.2.12 Sequential lipid extraction and quantification.

For lipid extraction, 0.5 g of stationary phase, lyophilised biomass was extracted in 75 ml of 2:1 (v/v) chloroform/methanol in 250 ml round bottom flasks at room temperature. Stirring was provided by a magnetic stirrer. At 45', 90', 135', 180', 24 h and, if necessary, 48 h the biomass was filtered through a filter paper, washed twice with chloroform/methanol and resuspended in fresh solvent, while the lipid-containing solvent was evaporated on a rotary evaporator, resuspended in a pre-weighed sample vial before being evaporated on a rotary evaporator and finally on a Schlenck line for 45 min or until no more weight loss was observed. Lipid was quantified in this manner by weighing the sample vials after solvent evaporation, at 45 min, 90 min, 135 min, 180 min, 24 h and 48 h or until an increase of less than 2% in lipid content was observed.

2.2.13 Elemental analysis and biochemical composition.

The microalgae samples were analysed by TGA for ash and moisture content on a TA Instruments IR5000Q TGA in air. The TGA oven temperature was increased to 105°C and held for 15 min, subsequently ramped to 550°C and held for 60 min to determine the moisture and ash content respectively. Ultimate analysis was determined by the C, H, N, S content of the samples using a CE Instruments Flash EA 1112 series elemental analyser. The results from the proximate analysis were used to calculate the elemental composition on a dry ash free basis.

The biochemical composition of the microalgae strains was determined by following techniques; for the protein analysis the a modified Lowry method by J. Waterborge was used (Waterborge, 2002) which involves the use of a folin reagent, subsequent absorbance measurements at 720 nm and comparison to a bovine standard absorbance at the same wavelength. Total carbohydrate content was determined by a sulphuric acid hydrolysis method (Gerchakov and Hatcher, 1972). The absorbance was measured at 485 nm and compared to a standard from glucose. Lipid extraction was performed using the Folch method employing a 2:1 methanol/chloroform extraction at 35°C, using an ultrasonic bath for 45 minutes. Approximately 100 mg of algae were treated with aliquots of 5 ml solvent mixture. After sonication, 1.7 ml of water was added to the samples, centrifuged at 3500 rpm (*g* force of 2264) for 10 minutes and the lipid containing chloroform layer recovered. The procedure was repeated with fresh solvent mixture. The lipids were finally quantified gravimetrically.

2.2.14 Hydrothermal liquefaction.

The hydrothermal processing was performed in custom built Swagelok reactors from 316 stainless steel 1.905 cm x 10 cm Swagelok pipes and are essentially closed, bomb type reactor tubes. The reactors had an internal volume of 25 ml. Using an internal K-Type thermocouple the time to reach

reaction temperature of the reactants was measured to be 2 min. The reactors were submerged completely into a fluidised sand bath (FSB-3, OMEGA Engineering Ltd, Manchester, UK) at the desired temperature and residence time. Once the desired residence time was reached the reactor was quenched in a cold water bath. Reactors were charged with approximately 1 g of microalgae and 10 ml of distilled water. 15 min were measured from the point the reactors were submerged into the hot sand bath, leading to an effective residence time of 13 min plus 2 min heat up time. All HTL reactions took place at 350°C ± 5°C.

Following liquefaction, two aliquots of each 10 ml dichloromethane (DCM) and 10 ml of water was added to the reaction mixture and the two phases separated in a separation funnel. The DCM phase was separated and filtered following which the solvent was removed by evaporation to determine the mass of bio-crude. The insoluble residue was weighed. A portion of the evaporated DCM solubles was analysed for C, H, N, S content and a portion by GC/MS. The bio-crude was analysed by GC/MS on an Agilent 5975B inert MSD. Separation was achieved on an Rtx 1701 60m capillary column, 0.32 id, 0.25 μm film thickness, using a temperature program of 40°C, hold time 2 minutes, ramped to 250°C, hold time 30 minutes, column head pressure of 30 psi. The aqueous phase following hydrothermal processing was centrifuged to recover additional solid residue and diluted to 250 ml with distilled water. Total organic and inorganic carbon (TOC, TIC) in the aqueous phase was determined using a TOC analyser (HACH- IL 550 TOC, Hach-Lange, Germany) using a differential method. Size exclusion chromatography of the lipids was carried out on a Perkin Elmer Series 200 HPLC instrument with a Varian PGel column of 30 cm length, 7.5 mm diameter, 3μm particle size and a THF mobile phase flow rate of 0.8 ml/min.

2.2.15 Transmission electron microscopy to assess cell wall thickness

Algal samples for TEM were cultured as described above. After 8 days for late exponential phase and 20 days for stationary phase, 10 ml of each algal culture were centrifuged and culture medium replaced with 0.1 M sodium cacodylate buffer pH 7.4 (2.14 g of sodium cacodylate in 40 ml of dH2O pH adjusted to 7.4 using HCl, made up to 50 ml with dH2O) and mixed well. Cells were then pelleted (12,000xg, 5 min in an Eppendorf 5415C centrifuge), the supernatant was removed and the cells were resuspended in fixative solution (2.5% gluteraldehyde, 2% paraformaldehyde in 0.1 M sodium cacodylate buffer pH 7.4 with 2.5 mM $CaCl_2$) and incubated overnight at 4 ºC.

Cells were subsequently pelleted, the fixative was removed and cells were washed three times with 0.1M sodium cacodylate buffer over 20min. The last wash solution was removed and replaced with aqueous 1% osmium tetroxide and 1% potassium ferrocyanide for 1h in fume hood at room temperature. Meanwhile 3% low melting point agarose in distilled water was prepared in a glass test tube and kept in water bath at 35ºC. Cells were then pelleted and washed in dH2O three times over 15min. Cells pelleted again, supernatant removed and pellet transferred to a water bath at 35ºC. Cells were then encapsulated in warm agarose, stirred with a cocktail stick and droppered onto cool glass slides to solidify. 0.5mm sections of the agar drops were cut and transferred to glass vials and stained in 2% aqueous uranyl acetate, for 1h in the dark.

Samples were dehydrated in increasing concentrations of acetone (twice over 15min at each concentration), 30, 50, 70, 95%. Samples then placed in 100% dry acetone, changing 4 times over 30min at room temperature. After dehydration samples infiltrated with TAAB low viscosity resin and 100% dry acetone (1:1) for 2h. Liquid mixture then removed and replaced with 100% resin and put under vacuum for 1h (or until acetone removed). Final liquid mixture removed and replaced with fresh resin and left overnight.

Samples then placed in moulds with fresh resin and polymerised in an oven at 70ºC for 24h. Samples were sectioned (~0.1µm thick) using a Reichert-Jung 'Ultracut E' Microtome, using a Diatome diamond knife (Knife number MS11872). Sections were supported on SIRA 300 mesh copper grids.

Transmission Electron Microscopy (TEM) images were taken at the University of Bath Bioimaging Suite with a JEOL JEM1200EXII transmission electron microscope (operating voltage of 120 kV) and images captured with Gatan Dualvision digital camera.

3 Improving *P. ellipsoidea* for biofuel production by random mutagenesis and screening.

3.1 Introduction.

3.1.1 Random mutagenesis as a strategy for improving microalgae for biofuel production.

The legal and public opinion considerations associated with the production of biofuels from GM algae pose a considerable challenge notwithstanding the challenge of developing tools for the genetic engineering algae and bridging the considerable gaps in our knowledge of microalgal biology. Random mutagenesis offers a potential solution to both of those problems as strains developed by random chemical or radiation mutagenesis are not considered GM and can be freely released in the environment with more than thousands of plant varieties having been released to date (Maluszynski *et al.*, 2000). Furthermore the random nature of this kind of genetic manipulation means that knowledge of the pathways and genes involved in the characteristic to be mutated is not necessary. Instead, a high-throughput and specificity screening method is needed to screen a large number of mutants and detect desirable characteristics among many random mutations. This approach has been used with some success in algae for increasing omega-3 lipid production in *P. lutheri* (Meireles *et al.*, 2003), astaxanthin content in *H.pluvialis* (Tripathi *et al.*, 2001), reducing the light-harvesting antenna of *Cyclotella* sp. (Huesemann *et al.*, 2009) and improved TAG accumulation in *Chlorella* sp. (Manandhar-Shrestha and Hildebrand, 2013).

If algae are to be actually improved by random mutagenesis they need to not only show the desired characteristic but also to be competitive in growth rates and/or overall productivity. Because of the random character of this method and the numerous mutations that will be present in each cell, mutants generated by random mutagenesis can show reduced growth rates

and fitness. This caveat is illustrated in Huesemann *et al.* (2009) where 10000 mutants were generated in the first round and 6000 in the second round of mutagenesis. These resulted in mutant strains with a reduced light-harvesting antenna which should show superior growth rates and biomass density under high light but when tested, they were found to be less productive than the wild-type. Manandhar-Shrestha and Hildebrand (2013) used fluorescence activated cell sorting (FACS) as a high throughput method to not only screen for high lipid mutants but also subject the mutants to directed evolution in order to quickly increase their fitness back to the levels of the wild-type while maintaining a higher lipid content.

3.1.2 Algaenan cell walls in the novel oleaginous alga Pseudochoticystis ellipsoidea.

P. ellipsoidea is a novel oleaginous green alga recently isolated from a hot spring in Japan by Satoh *et al.* (2010). It is a fast growing, acidophilic alga that accumulates approximately 30% dw of lipids under nitrogen starvation. Previous work in our lab established that this alga produces an algaenan cell wall (Smith-Baedorf, 2012) (fig. 3.1.). Algaenan is a non-hydrolysable heteropolyester comprised of very long chain (average length C:40), saturated or monounsaturated hydroxy-alcohols, carboxylic acids, dicarboxylic acids and diols among others mainly linked by ester bonds (Allard and Templier, 2001; Allard *et al.*, 2002; Blokker *et al.*, 2006, 1998a).

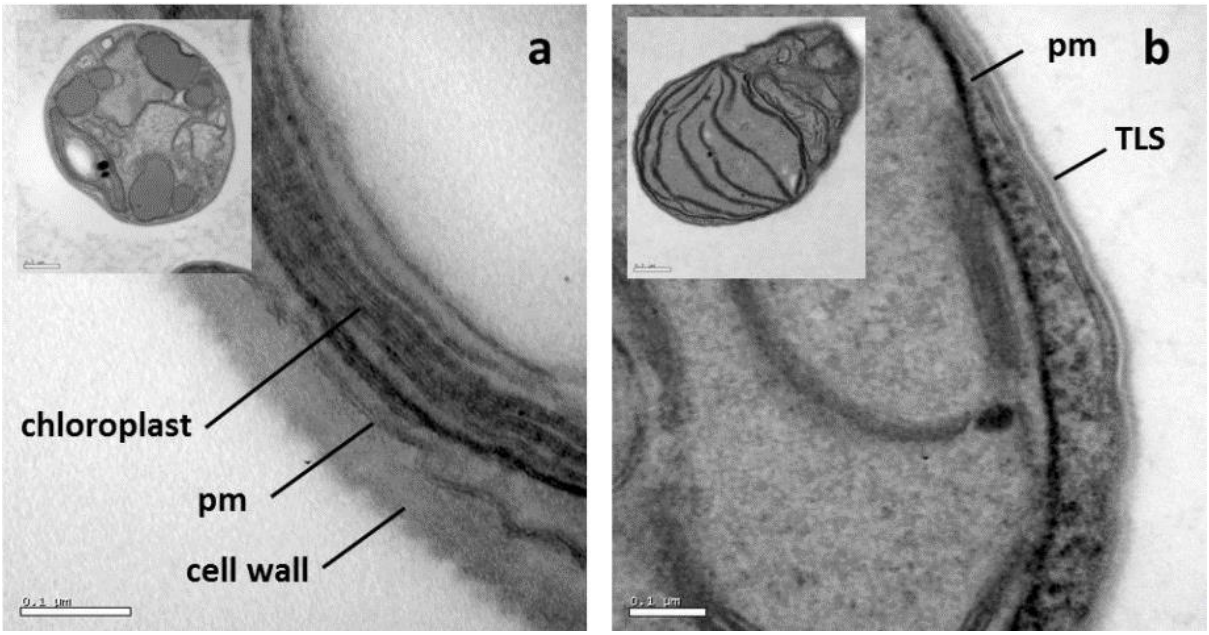

Figure 3.1. TEM images comparing the cell walls of *C. vulgaris* (a) and *P. ellipsoidea* (b).

Note the presence of a thick polysaccharide-based cell wall in (a) and the presence of a "trilaminar" algaenan cell wall in (b) as well as absence of a visible polysaccharide layer. "pm" = plasma membrane and "TLS" = trilaminar structure (algaenan). Scale bar = 0.1µm. Adapted from Smith-Baedorf, (2012).

Algaenan has been cited in the literature as being extremely resistant to enzymatic attack (Atkinson *et al.*, 1972; Smith-Baedorf, 2012), non-hydrolysable by strong acids, bases and organic solvents (Allard *et al.*, 1998) as well as impermeable to various stains (Smith-Baedorf, 2012; Zych *et al.*, 2009).

Because of the resistant nature of algaenan there is a case for the genetic modification of the *P. ellipsoidea* cell wall. Because relatively little is known about the biosynthesis of this cell wall polymer (see section 1.5.3) and because of the restrictions imposed on GM organisms, random mutagenesis would be the ideal strategy for cell wall modification in this species. Although a lengthy chemical extraction is required to prove the presence of algaenan, Smith-Baedorf (2012) showed that the fluorescent dye Calcofluor white can be used to detect the presence of algaenan with reasonable confidence (fig. 3.2.). Because of the ability of this stain to label common cell wall polysaccharides such as ß-1, 3 and ß-1, 4 glucans as well as chitin and glucosamine, but not

algaenan, it can be exploited along with FACS to provide a high-throughput method of screening for cell wall mutants.

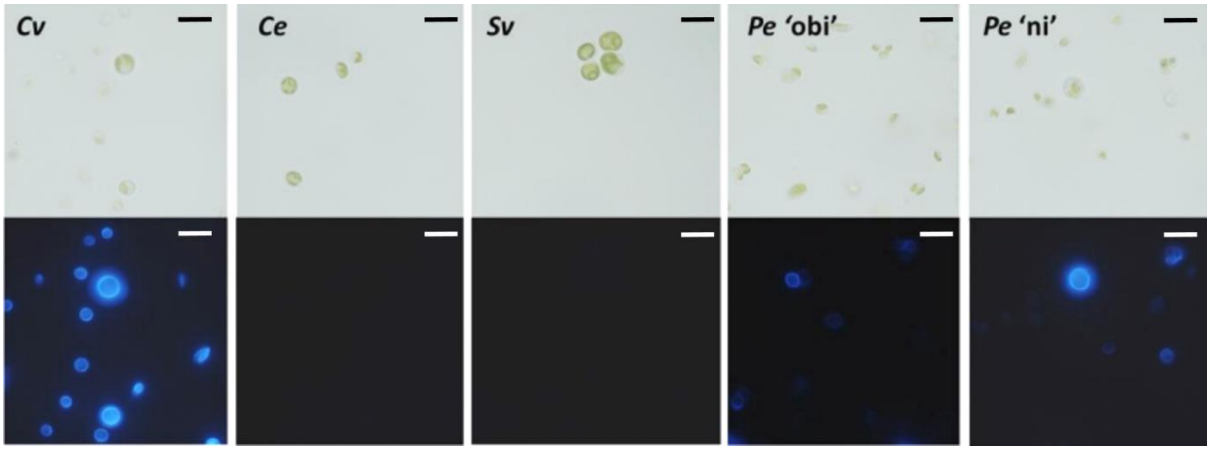

Figure 3.2. Light microscopy (top) and fluorescence microscopy (bottom) of five microalgal strains (from left to right); *C. vulgaris, C. emersonii, S. vacuolatus and P. ellipsoidea* 'Obi' and 'Ni' stained with calcofluor. Top and bottom images for each species were taken from the same field of view. *C. vulgaris* is the only strain of the above that does not produce an algaenan cell wall and the only one that shows 100 % staining with calcofluor. Scale bar = 20μm.

3.1.3 Scope of this chapter.

In this study random mutagenesis by UV radiation and EMS as well as FACS for the high throughput screening of mutants are being developed for the novel oleaginous alga *Pseudochricystis ellipsoidea* with the ultimate aim of isolating mutants with improved characteristics for biofuel production. Specifically, we aimed to isolate cell wall deficient mutants as well as determining the genetic and biochemical basis of beneficial mutations. Furthermore, we aimed to isolate chlorophyll deficient (colour) mutants in order to identify a strain with a truncated light-harvesting antenna. A growth system for production of large amounts of algal biomass for the realistic evaluation of isolated mutants was also developed alongside mutagenesis methods (described in detail in chapter 4).

3.2 Methods and results.

3.2.1 Choosing a strain for mutagenesis.

Initially, two strains of *P. ellipsoidea*, termed Ni and Obi were made available by DENSO. An initial growth experiment was performed to assess the growth rates, biomass productivities and lipid accumulation. This was done by haemocytometer cell counts from microscopy pictures, assessment of dry weight, lipid staining with Nile Red and confocal microscopy as well as visual assessment. Strain Obi showed the highest cell densities (fig. 3.3.A) but strain Ni showed the highest biomass productivities (fig. 3.3.B). Furthermore, strain Ni showed larger cells and more lipid fluorescence per cell (fig. 3.4.) which is desirable for biofuel production as it makes algae more harvestable (Uduman *et al.*, 2010). After consultation with our sponsors (Hiroaki Fukuda, personal communication) it was agreed to focus our efforts on stain Ni as it was generally agreed to be the strain with the best biofuel potential.

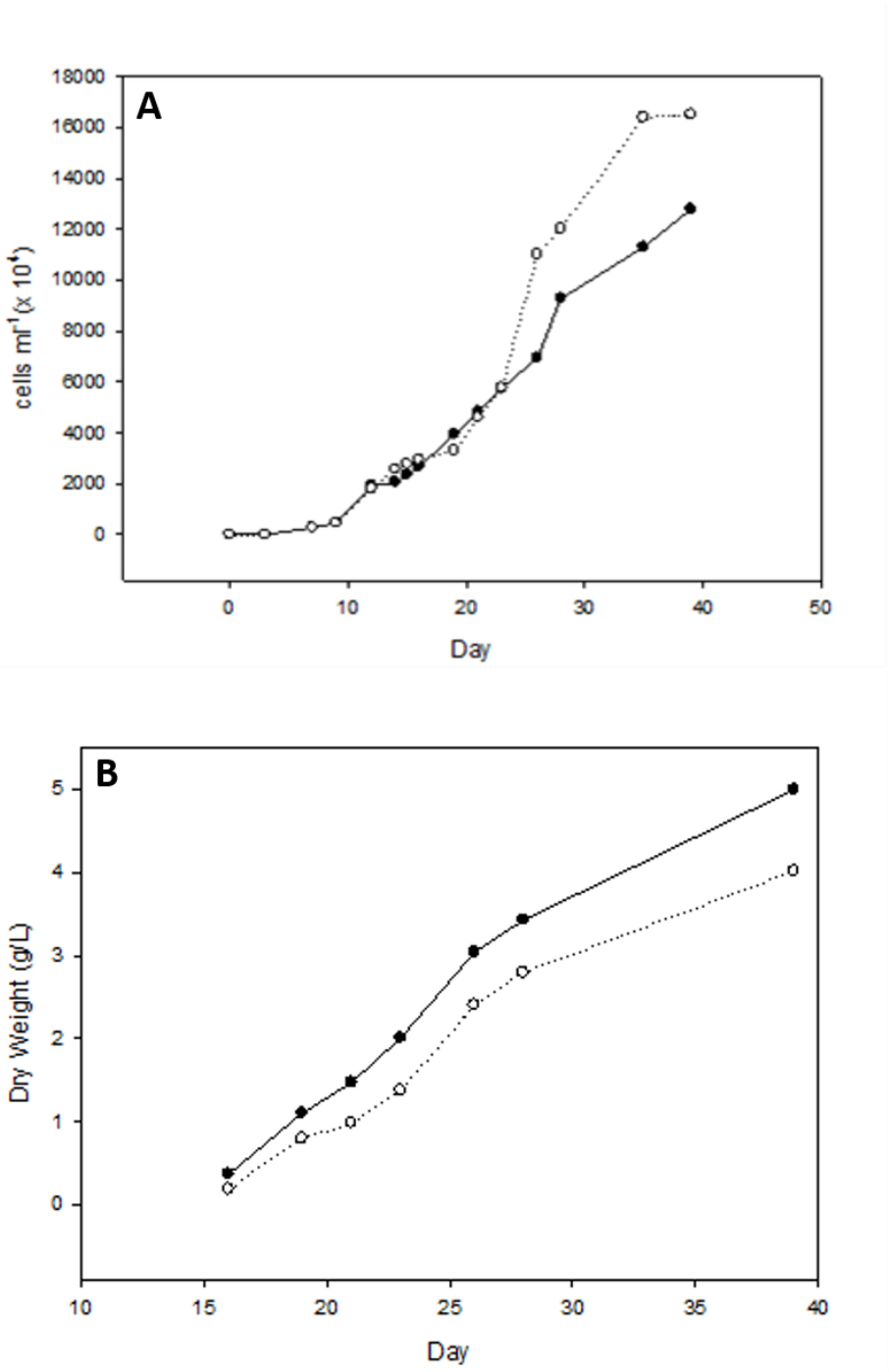

Figure 3.3. Growth of two strains of *P. ellipsoidea* in 100 ml shaken flasks expressed as; cell concentration (A) and dry weight (B). Strain Ni is represented by filled circles and strain Obi by empty circles. Data points represent averages of two measurements from the same culture flask (n=1).

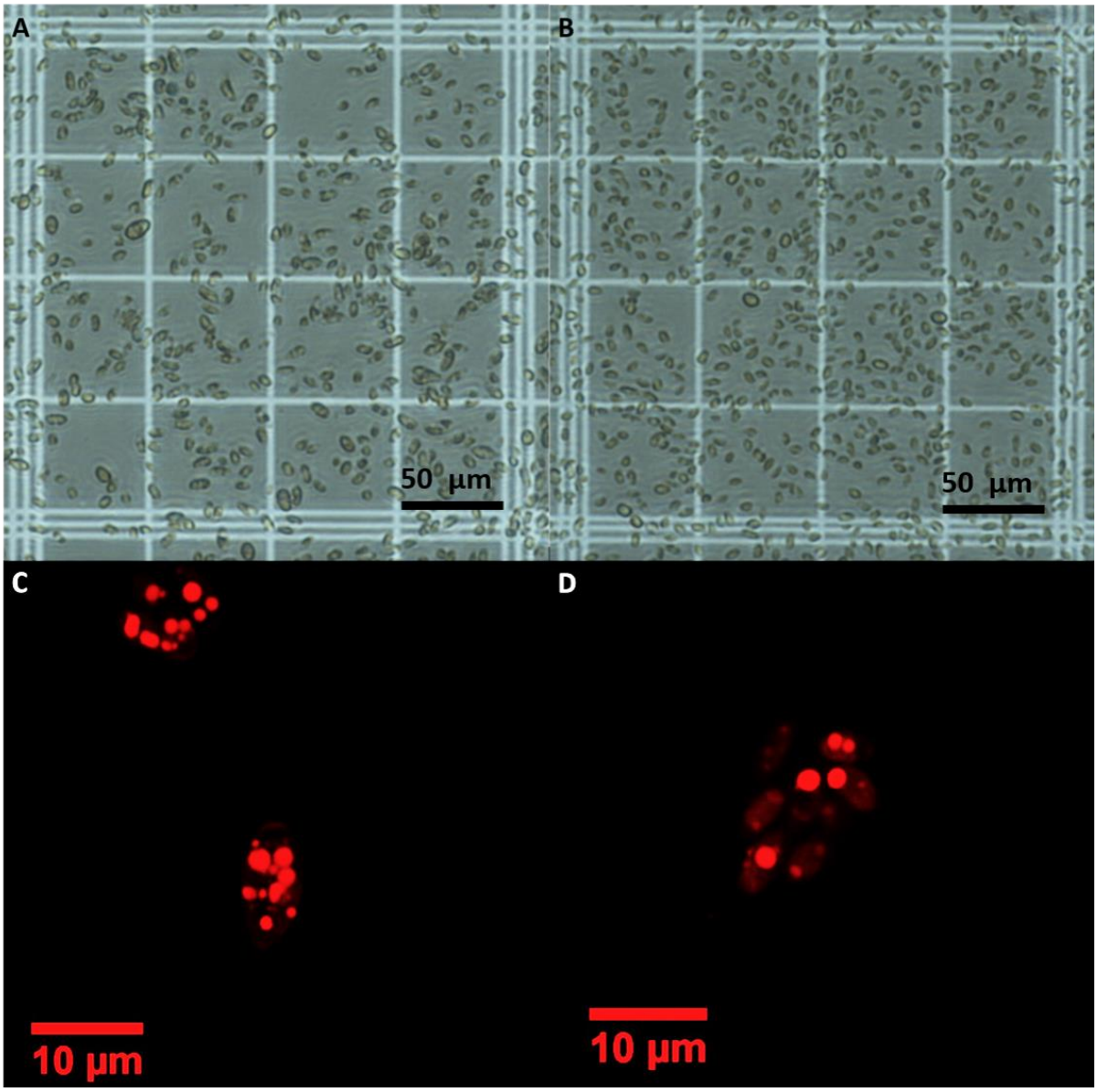

Figure 3.4. Brightfield microscopy (top) and confocal microscopy (bottom) of two *P. ellipsoidea* strains; Ni (A, C) and Obi (B, D) on day 28 of culturing. Lipids were stained with Nile Red and are visible as prominent red droplets. The difference in average cell size between the two strains is apparent in the brightfield images (top). Very faint chlorophyll autofluorescence is visible in the background. Brightfield and confocal images for each strain were taken from the same sample used to perform cell counts for day 28 (fig. 3.3A). Note the absence of lipid droplets in four out of eight cells present in strain Obi (D).

3.2.2 Developing a random mutagenesis protocol for P. ellispoidea.

The initial protocol used for UV mutagenesis was developed by Baedorf-Smith (2012). Briefly, petri dishes containing 25 ml of *P. ellipsoidea* cultures harvested or diluted to a density of 10^6 cells ml^{-1} were placed under a UV light source. At specific time points the cultures were agitated by

withdrawing and expelling a 1 ml sample five times using a pipettor and a 1 ml sample was subsequently taken. This was appropriately diluted and plated onto 1% agar-BBM plates that were incubated for 3-4 weeks at 25 °C and at 150 µmol photons m^{-2} s^{-1} PAR. Initially both strains of *P. ellipsoidea* were tested. Survival was calculated by counting agar plate colonies for each exposure (n=3) and converting those counts to percent survival by assuming the control plates showed an average of 100% survival. The calibration curves were done in order to find the point of 90% mortality. This is the point at which subsequent mutageneses would be performed as it ensures that survivors are indeed mutants and not wild-type cells. An exponential curve was fitted to the each curve and the point of 10 % survival was calculated from the regression equation and rounded to the nearest minute. For strain Ni, the UV exposure required for 10% survival was 15 min (r^2=0.985) and for strain Obi 24 min (r^2=0.867) (fig. 3.5.). Because strain Ni showed a higher r^2 as well as better productivity, lipid content and larger cells (as described in the previous section), it was the strain used for all experiments henceforth.

Ethyl methanesulphonate (EMS) was also used for the random mutagenesis of *P. ellipsoidea*. The first attempt to a calibration curve for mutagenesis (60 min exposure) yielded an increase of colonies from the control (no exposure to EMS) before a slight decreasing trend was observed and at 60 min of EMS exposure the colony counts were still higher than the control.

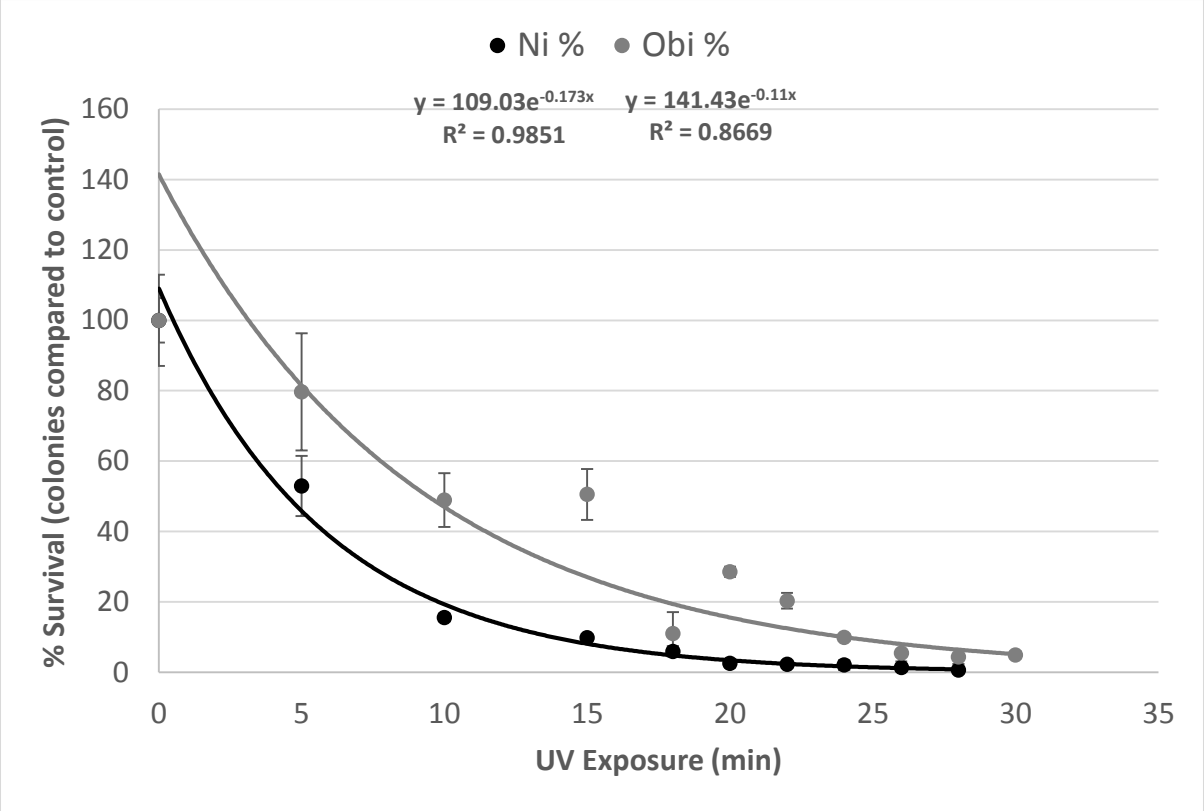

Figure 3.5. UV mutagenesis calibration and exponential fitted curves for *P. ellipsoidea* strains Ni (black line) and Obi (grey line) by colony counts on 1% agar plates. Data points represent mean % survival of algae assessed by colony counts on three replicate agar plates (n=3) from a single mutagenesis performed simultaneously for both strains. Error bars represent ±1 SE. Exponential curve equations and R^2 values for each strain are displayed below the legend.

This could not be explained so a new batch of EMS was ordered, the test tubes were covered in aluminium foil to prevent the action of cellular photo-induced DNA repair and the exposure was increased to 180 min. No increase from the control was observed in the second run but 180 min of exposure was still not sufficient to induce 90% mortality. A third calibration curve was performed, this time increasing the exposure to 240 min. On this calibration curve the exposure to EMS needed to induce 90% mortality was found to be 150 min (r^2=0.81). This meant that the sensitivity of the cells to EMS had changed since 150 min of EMS exposure only induced a mortality rate between 40% and 50%. As none of the calibration curves grouped with another a fourth calibration curve had to be undertaken. This, once again showed an increase in colony counts compared to the control for the first two sampling points and then a very steep decrease, essentially from 97.2%

survival to 0% survival within 30 minutes (120 min to 150 min of EMS exposure). The above calibration curves are summarised in fig. 3.6.

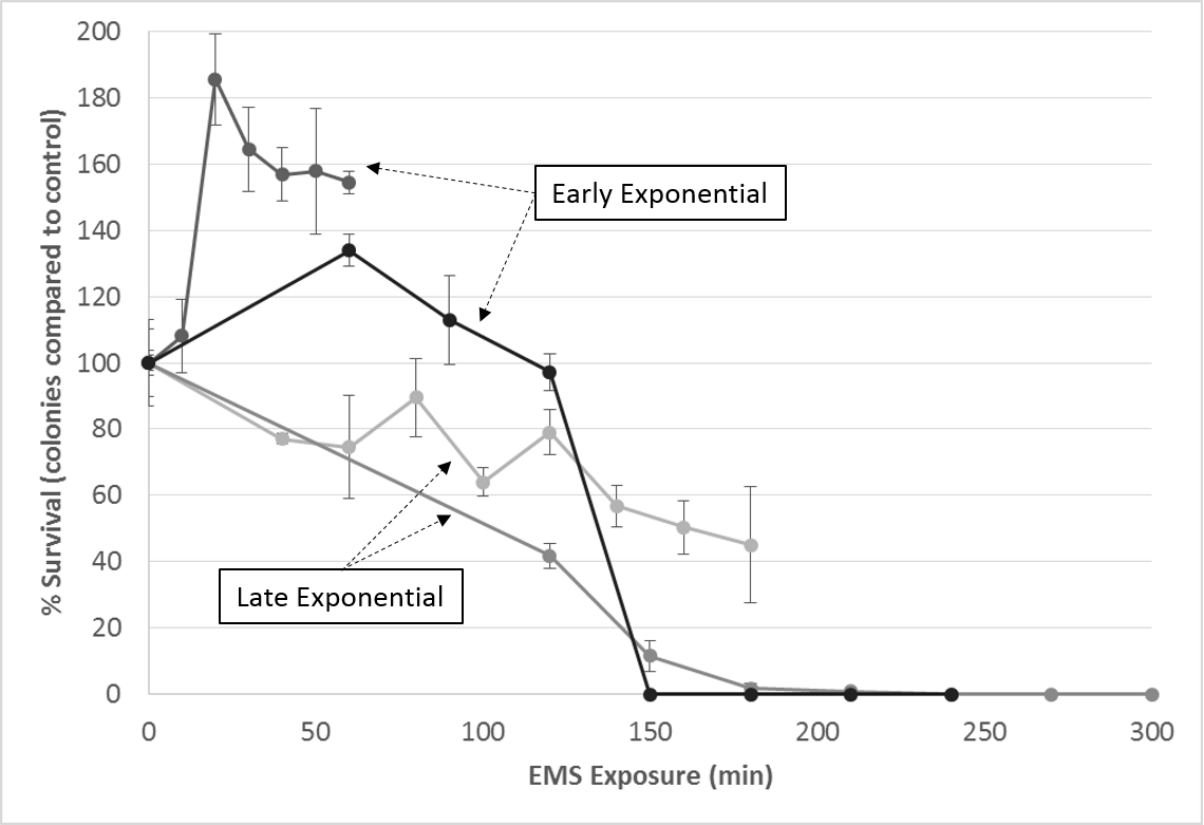

Figure 3.6. Summary of EMS mutagenesis calibration curves on *P. ellipsoidea* Ni showing the influence of growth stage and the general variability on the sensitivity of this alga to EMS. Survival measured by colony counts on 1% agar plates. Error bars represent ±1 SE, n=3.

When looking at the first four attempts of a mutagenesis calibration curve with EMS it was apparent that there was considerable variability in the sensitivity to the mutagen but also an unexplained increase in colony counts compared to the control. However this happened in only two out of four curves. To try and understand the source of variability, we looked back at the cell counts of our *P. ellipsoidea* cultures and calculated the plating efficiency in comparison to the observed number of colonies (table 3.1.). The two mutagenesis events that showed an increase in colony counts compared to the control (25/05 and 26/08) were both performed at early exponential stage cultures (~10^6 cells ml^{-1}). Furthermore the plating efficiency at t=0 min was 58.1% and 75.4% and showed a maximum of 107.75% and 100.61% at

EMS exposures of 20 min and 60 min respectively. In contrast the two mutagenesis events that did not show an increase in colony counts in comparison to the control (15/06 and 06/07) were performed on cultures on late exponential stage cultures with cell densities of $9.65*10^6$ cells ml^{-1} and $13.875*10^6$ cells ml^{-1} respectively. Plating efficiencies for these two mutagenesis events were 87.93% and 67.78% respectively (table 1).

Table 3.1. Summary of EMS calibration attempts. Plating efficiency was calculated as the number of colonies observed in comparison to the number of colonies expected from the cell counts prior to mutagenesis.

Date	Cell Density before dilution (*10^6 cells/ml)	Plating Efficiency (%)	Max. Plating Efficiency (%)
25/05/2011	1	58.10	107.75
15/06/2011	9.65	87.93	87.93
06/07/2011	13.875	67.78	67.78
26/08/2011	0.87	75.40	100.61

When looking at the microscope pictures of typical *P. ellipsoidea* growth curves it became clear that *P. ellipsoidea* Ni tends to grow in aggregates of cells in early exponential stage (fig. 3.7.A) but is present as single cells in late exponential and stationary stage (fig. 3.7.B). The cell density at which *P. ellipsoidea* changes appearance in our cultures was around 5-10* 10^6 cells ml^{-1}, therefore our four mutagenesis attempts with EMS can be grouped into two categories which we have named early exponential and late exponential.

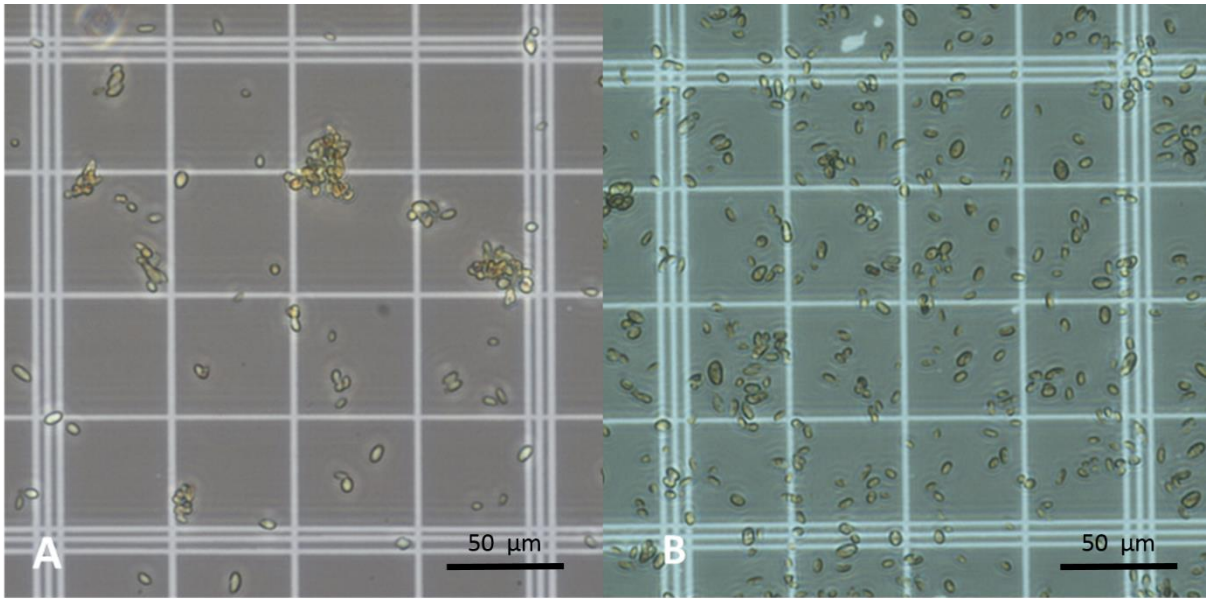

Figure 3.7. Light microscopy images showing the typical appearance of *P. ellipsoidea* Ni at different growth stages. Cells present mainly in aggregates at early exponential stage (left, day 12) and mainly as single cells in late exponential (right, day 16).

From an inoculation density of $0.05*10^6$ cells ml^{-1}, it took *P. ellipsoidea* 5-8 days to reach the desired cell densities ($1\text{-}3*10^6$ cells ml^{-1}). The change in appearance from aggregates to single cells was also accompanied by a change in the division rates. These were generally around 0.4-1.2 divisions day^{-1} in early exponential and < 0.3 divisions day^{-1} in late exponential (fig. 3.8.).

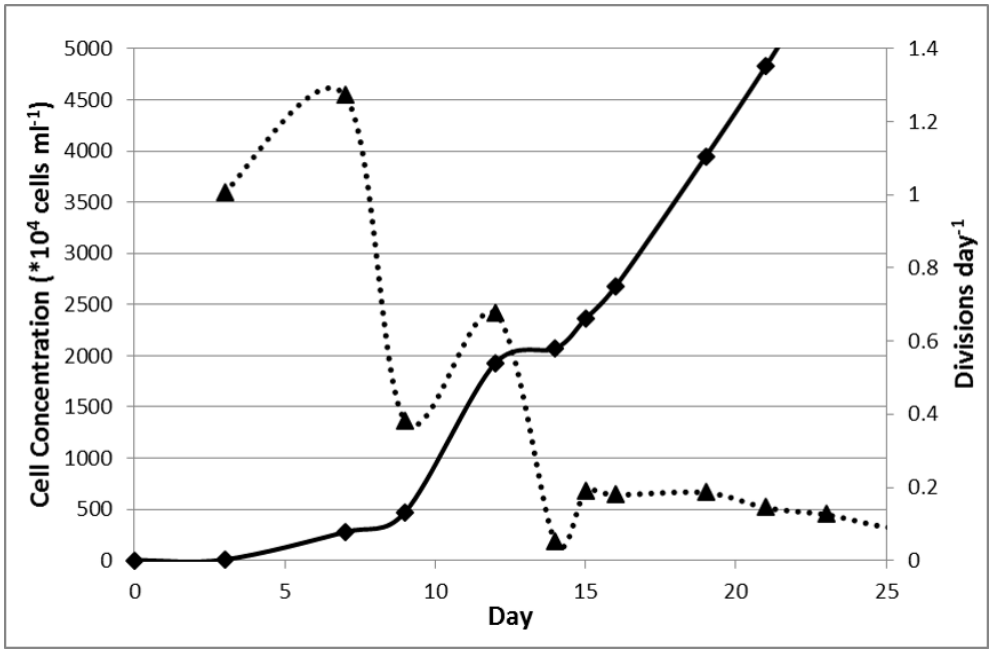

Figure 3.8. Typical cell counts (black line) and division rates (dashed line) of *P. ellipsoidea* Ni grown for mutagenesis.

The characteristics of *P. ellipsoidea* Ni described above, presented a challenge for mutagenesis. To make matters even more complicated, at that stage of the project (September 2011), numerous attempts of obtaining UV mutants at the predetermined exposure (15 min) yielded very variable survival rates (5-50%) and neither direct plating nor FACS screening was able to identify any pigment mutants. As pigment mutants are easy to visually identify and a relatively common type of mutant in algal studies, this also suggested there was something wrong with the methods.

It was concluded that the presence of aggregates at early exponential stage was undesirable for mutagenesis. The presence of aggregates tended to distort the colony counts by showing increases in colony numbers at mutagen exposures of t>0 min. It also meant that the response of the algae to the mutagen is extremely variable as the size of aggregates and how that changes day to day is fairly unpredictable. Furthermore, the presence of aggregates made it impossible to administer an equal dose of mutagen to all cells in a culture as cells in the centre of clusters would be protected and thus even at 90% mortality it was not certain that the colonies appearing on agar plates

were not in fact wild-type cells. This would certainly explain the lack of pigment mutants after having visually screened thousands of colonies and FACS screened tens of thousands of cells.

A choice had to be made at that point on how to progress. Cells could either be mutagenised in late exponential stage where no or very few cell aggregates were present. This would have the benefit of giving more replicable results as we were able to get calibration curves from that growth stage (fig. 3.5. and fig. 3.6.). Mutagenising at late exponential stage though, would have the disadvantage of reduced plating efficiency but more importantly reduced cell division rates. Performing mutagenesis when cells have high rates of division is beneficial because expressed genes are more likely to be mutated and the genes targeted in the experiments described here are genes involved in cell wall and chlorophyll synthesis and therefore would be more highly expressed in early exponential stage cells.

Considering the above it was decided that a non-lethal method to separate the algal aggregates had to be developed to enable mutagenesis of early exponential *P. ellipsoidea*. A number of methods were trialled such as vortexing with or without the addition of sand, glass beads and surfactants such at Triton-X100 and sodium dodecyl sulphate (SDS) as well as gentle sonication in a sonic bath. This last method was the only one that showed some promise and was therefore investigated further.

Briefly, 1.5 ml Eppendorf microcentrifuge tubes containing 1 ml of early exponential stage *P. ellipsoidea* were floated in a sonic bath using a foam tube rack so that the full volume of culture in the tube was submerged in the sonic bath. These were sonicated and sampled at intervals of 5 min. Cell concentrations of samples were measured by flow cytometry. The tubes were then left in the dark for 24-hours unshaken and recounted the next day after brief vortexing (3 s). Visual assessment by optical microscopy was also performed.

The apparent viable cell density increased significantly in all sonic treatments when counted immediately after sonication (fig. 3.9.) approximately doubling from 5.9 10^3 cells ml^{-1} to 11-14 *10^3 cells ml^{-1}. After 24 hours in the dark the same tubes were recounted. Most sonic treatments showed similar values to the control with a range of 5-8.1 *10^3 cells ml^{-1}, indicating that the cells were re-aggregating or losing viability.

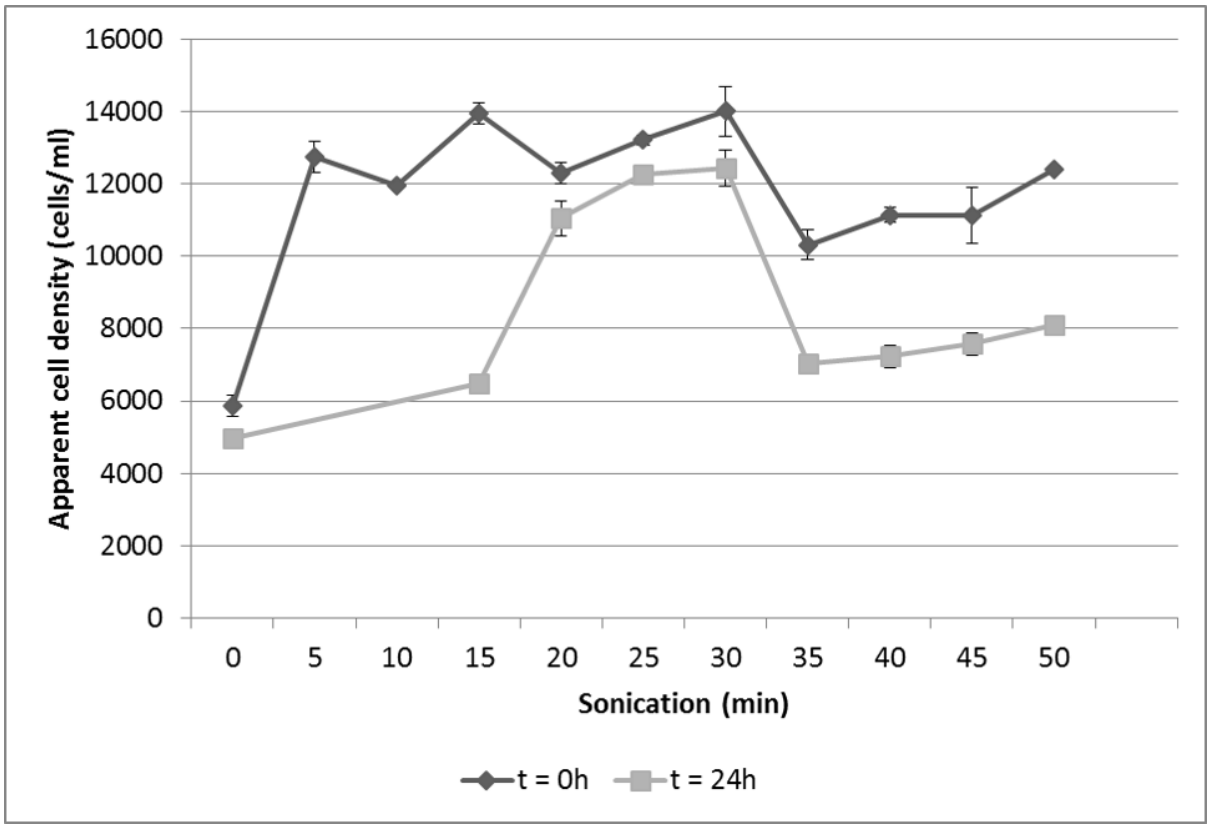

Figure 3.9. Treatment of exponential stage *P. ellipsoidea* Ni in a sonic bath and its effect on the apparent cell concentration as measured by flow cytometry immediately after (t=0, dark grey) and 24 hours after sonication for different time periods. Note the period between 20 and 30 minutes of sonic treatment where algal aggregates were effectively disrupted and the apparent number of cells stayed the same after 24 h (i.e. cells did not re-aggregate). Error bars represent ±1 SE, n=3.

However when exposed to the sonic bath for 20-30 min, *P. ellipsoidea* cells after 24 hours unshaken had still very similar apparent viable cell densities with two out of three measurements being not significantly different to each other with p=0.055, p=0.023 and p=0.078 for the 20, 25 and 30 min treatments respectively. Thus sonic treatment for 20-30 minutes was shown

to be effective at breaking cell aggregates without affecting their viability and without the treated cells re-aggregating after 24 hours. From this trial, 20 minutes of sonic treatment was chosen to be used in subsequent mutagenesis efforts as it was the gentlest treatment that would give the desirable results.

3.2.3 FACS screening.

After mutagenesis the remaining algal cultures that were not inoculated in agar plates, were placed in 50 ml conical flasks, supplemented with 0.1% acetate and returned to the growth chamber for 24 h in the dark (covered in aluminium foil) and 48-72 h in the light (150 µmol photons m^{-2} s^{-1} PAR) before taken to the FACS for screening. This delay was done in order to give the algal cells enough time to divide and express mutations. *P. ellipsoidea* was screened on two different parameters; chlorophyll autofluorescence in order to isolate putative light-harvesting mutants and calcofluor fluorescence in order to isolate cell wall mutants. The parameters were set using wild-type populations as a control.

Chlorophyll autofluorescence was found to vary by more than an order of magnitude in wild-type populations. This meant that any putative light-harvesting mutants would be found in the lower end of the natural range of chlorophyll fluorescence. Therefore FACS could not be used to directly identify photosynthetic mutants but to eliminate a high percentage (>90%) of cells that were certainly not pigment mutants. UV mutagenised populations of *P. ellipsoidea* showed two distinct populations of cells separated by an approximately 100-fold difference in chlorophyll autofluorescence (fig. 3.10.). It was assumed that the low chlorophyll population was the group of cells that had been killed by UV radiation during mutagenesis. Two sorting gates were set for FACS isolation for putative mutants; one to isolate the low-fluorescing cells from the high chlorophyll (putative viable) population and the other to isolate the highest fluorescing cells from the low chlorophyll

population (putative dead). It was confirmed by agar plating and liquid cultures that the low-chlorophyll population contained no viable cells.

The sorted cells within the viable population did not reveal any pigment/pale mutants in three different mutagenesis and sorting events. This was probably related to challenges in developing a good mutagenesis protocol for *P. ellipsoidea* as described in the above section.

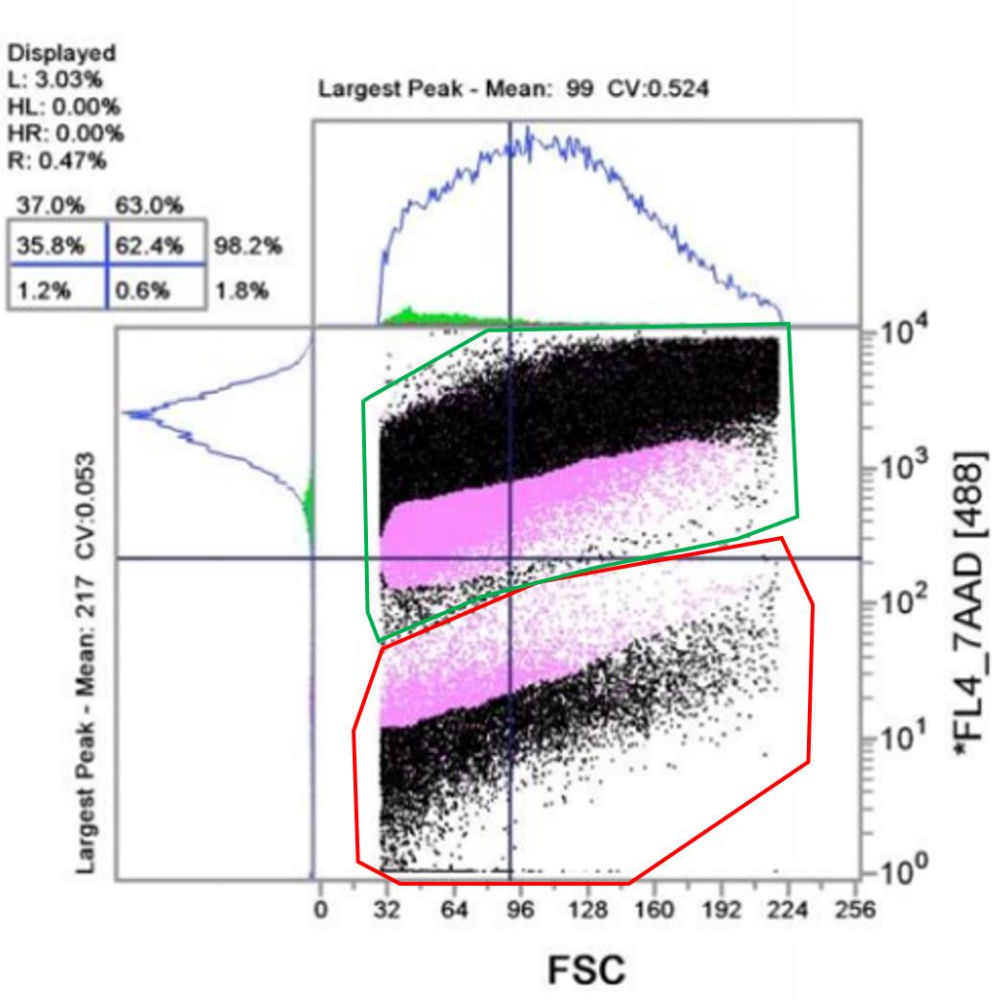

Figure 3.10. FACS screening of UV mutagenised *P. ellipsoidea* for light-harvesting mutants. The x-axis represents forward scatter (proxy for cell size) and the y-axis chlorophyll fluorescence. The green polygon highlights the viable proportion of the population and the red polygon highlights the dead proportion of the population. The pink coloured areas within each polygon represent the sorting gates used for the isolation of mutants.

Calcofluor staining and fluorescence microscopy of *P. ellipsoidea* revealed that only a minor proportion (approximately 5-50%) of wild-type cells showed calcofluor fluorescence. Calcofluor-positive cells within the wild-type population are most likely the cells that are actively dividing, damaged or dead and therefore have gaps in the algaenan layer of their cell wall where calcofluor can penetrate and bind to the cellulose layer below (Smith-Baedorf, 2012; Zych *et al.*, 2009). This meant that, as with the screening for photosynthetic mutants above, FACS could not be used to directly detect mutants but only as a step to increase the throughput by eliminating 70-95% of cells that were certainly negative for calcofluor staining. UV mutagenised populations of *P. ellipsoidea* were present as two distinct populations distinguished by their chlorophyll fluorescence as viable and dead and also by their calcofluor fluorescence as calcofluor positive and calcofluor negative (fig. 3.11.). Calcofluor positive cells were sorted in two different gates termed Calc+ and Calc++, plated in agar plates, and transferred to 96-well plates for screening using a plate reader. The remainder of the liquid culture was grown in BBM and assessed by a second round of FACS to reveal any changes in the calcofluor fluorescence of the population.

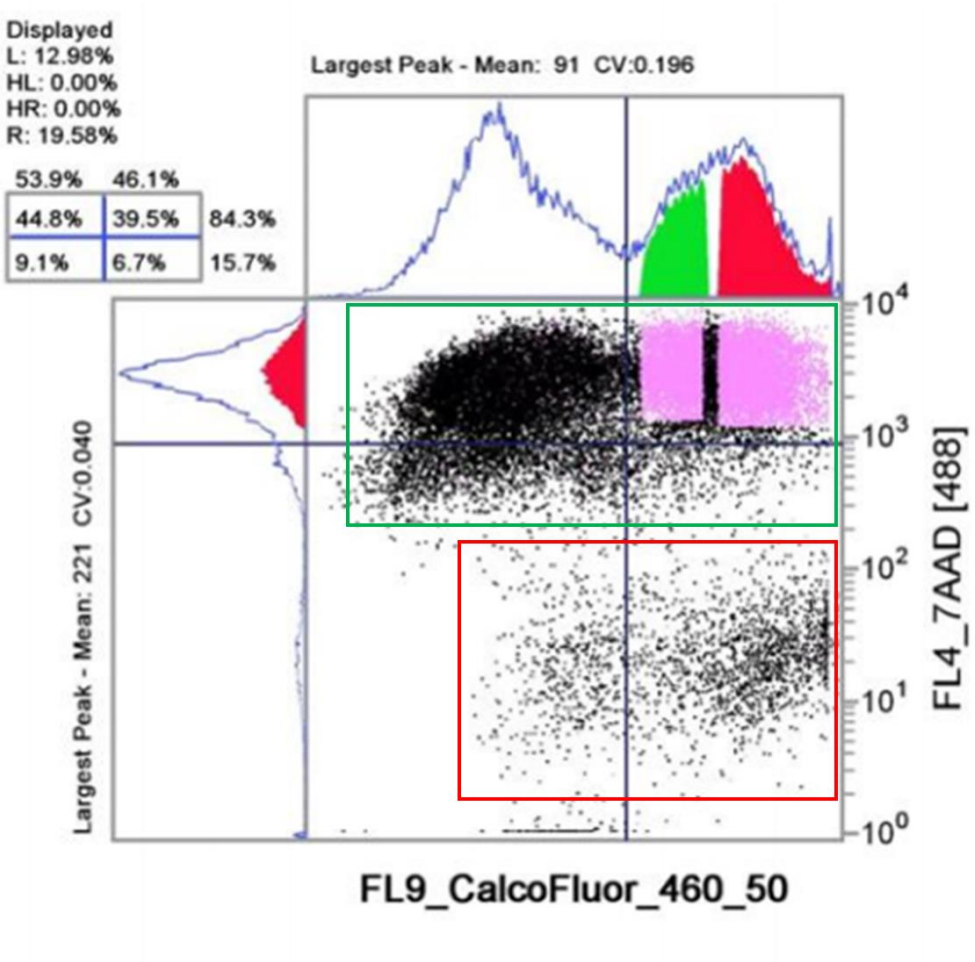

Figure 3.11. FACS screening of UV mutagenised *P. ellipsoidea* for cell wall mutants. The x-axis represents calcofluor fluorescence and the y-axis chlorophyll fluorescence. The green square depicts viable cells and the red square dead cells. The two pink areas within the viable population represent the two sorting gates named Calc+ and Calc++.

Subsequent FACS screening did not reveal any changes in the calcofluor fluorescence of the population either in percentage or in the intensity of fluorescence, therefore we concluded that none of our calcofluor positive cells were truly calcofluor positive mutants.

3.2.4 Final mutagenesis protocol and isolation of mutants.

As discussed above, after the first year of mutagenesis attempts, several challenges had to still be overcome. The main challenge was the apparent variability of *P. ellipsoidea* in its tolerance to mutagens depending

on growth stage, where a target of 10% survival resulted in 5%-50% survival. The conspicuous absence of pigment mutants throughout the mutagenesis efforts also suggested that the mutagenesis methods needed improving. As described above one of the main problems identified was the growth of *P. ellipsoidea* in aggregates in the early exponential growth stage resulting in an uneven dosing of the mutagen among cells meaning that wild-type cells were more likely to be present than desired.

Because of the above results, the mutagenesis protocols were modified in order to standardise the timing of mutagenesis, eliminate cell aggregates in early exponential growth stage and minimise exposure to light, especially blue light which induces DNA repair by photoreactivation (Cleaver, 2003; Sinha and Häder, 2002; Vlcek *et al.*, 1995). In particular, cells were monitored carefully in order to perform mutagenesis at a density of $1\text{-}3*10^6$ cells ml^{-1}. Gentle sonication for 20 min was performed prior to mutagenesis in order to eliminate cell aggregates. Two magnetic stirrers were placed inside the UV cabinet so that the petri dishes containing the cultures were continuously stirred while being exposed to UV radiation. The UV cabinet was covered with aluminium foil so that mutagenesis could take place in the dark. The samples taken from UV and EMS mutagenesis were placed in red/orange microcentrifuge tubes to minimise exposure to blue light which induces photoreactivation. These were diluted and spread on agar plates under low light and immediately returned to the dark. An outline of the key events in our mutagenesis and screening methods is shown in fig. 3.12.

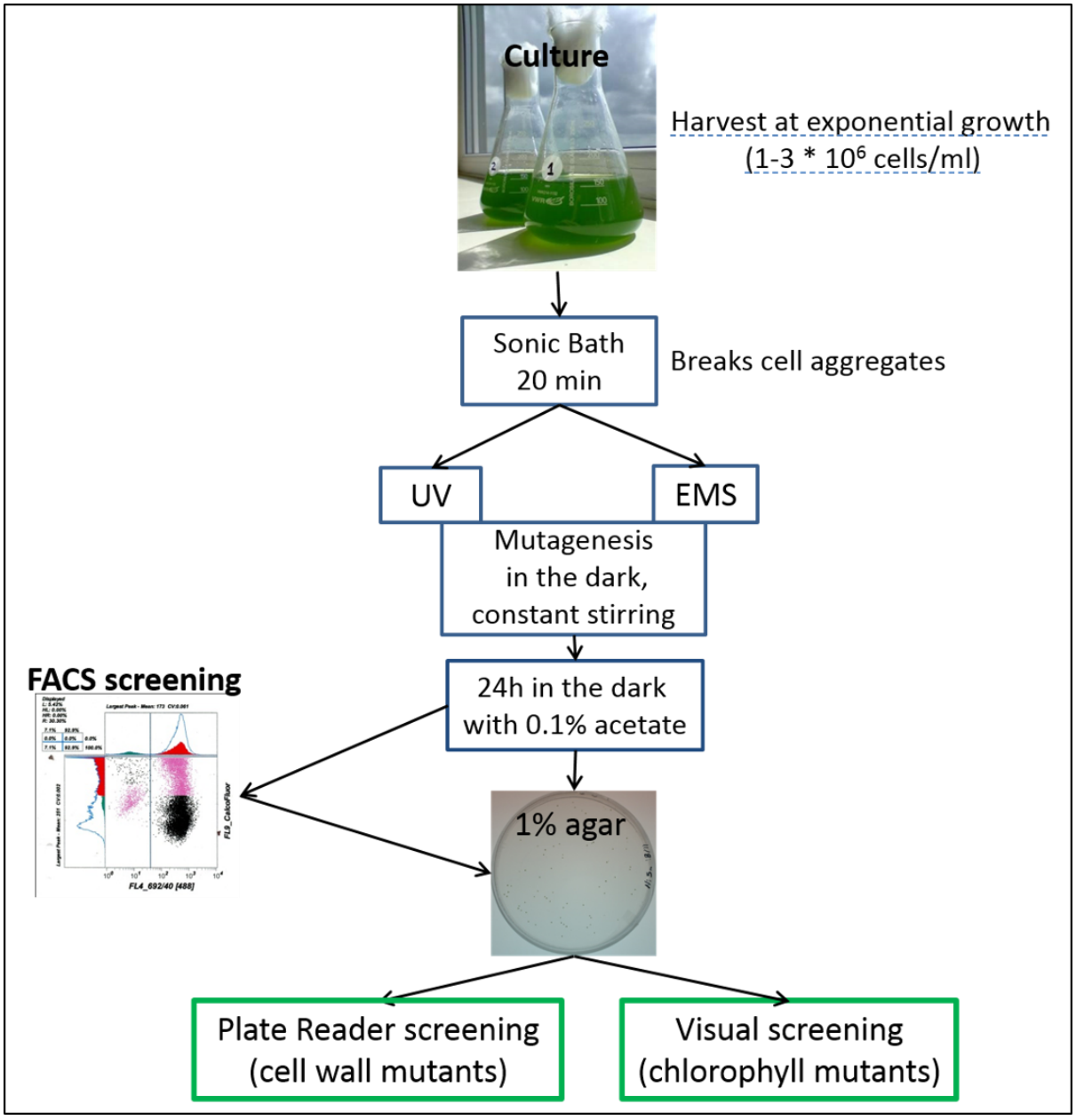

Figure 3.12. Diagram of the finalised mutagenesis and screening process.

A UV mutagenesis was done in duplicate as described above with exposure up to 50 min. The first calibration curves showed extremely reduced plating efficiency for the control treatment (7.8%) and no colonies in most of the UV-exposed treatments (fig. 3.13.).

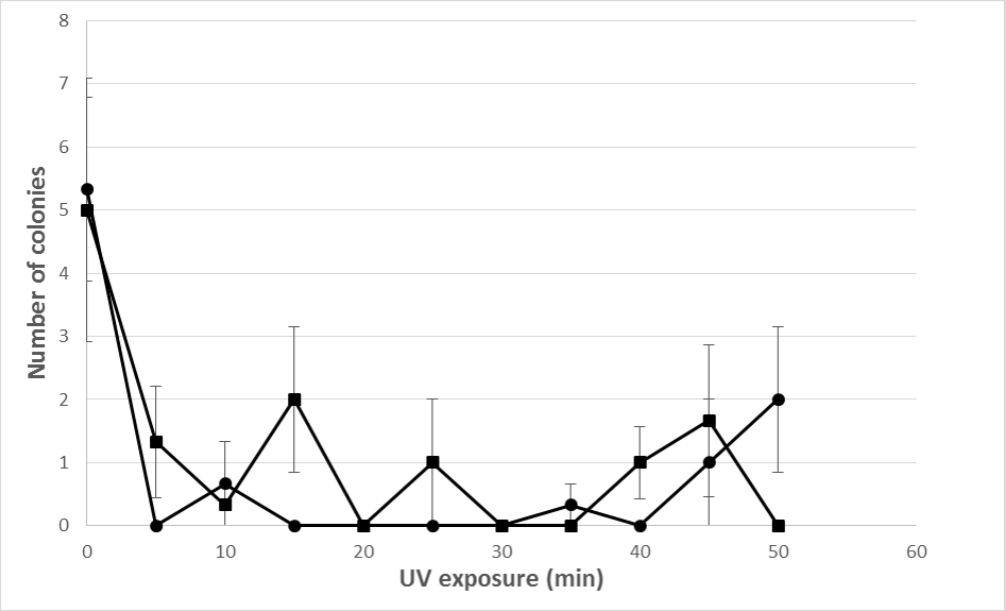

Figure 3.13. UV mutagenesis calibration curves from two replicate mutagenesis events. Note the low number of colonies in the control (UV exposure = 0) where the expected number of colonies was 66. Error bars represent ± 1 SE (n=3).

It was assumed that the sonic treatment, while not significantly affecting viability in the short term (fig 3.9.), had an adverse effect on the plating efficiency of *P. ellipsoidea*. Furthermore, evidence from the literature suggests that sonication can make cells more sensitive to UV radiation (Joyce and Mason, 2008). The effect of sonic treatment on the plating efficiency of *P. ellipsoidea* meant that we could not perform mutagenesis at the early exponential growth stage.

As a final test of the mutagenesis protocols, we performed UV mutagenesis as described above with the species *Chlorella emersonii*, a green alga commonly handled in our lab that also possesses an algaenan cell wall but does not show cell aggregates in any of its growth stages. We omitted sonic pre-treatment for this species but otherwise carried out UV mutagenesis in the manner described above. We were able to isolate a pigment mutant (pale) from a single mutagenesis event with this species, confirming that there were no basic errors in the mutagenesis method (fig. 3.14.).

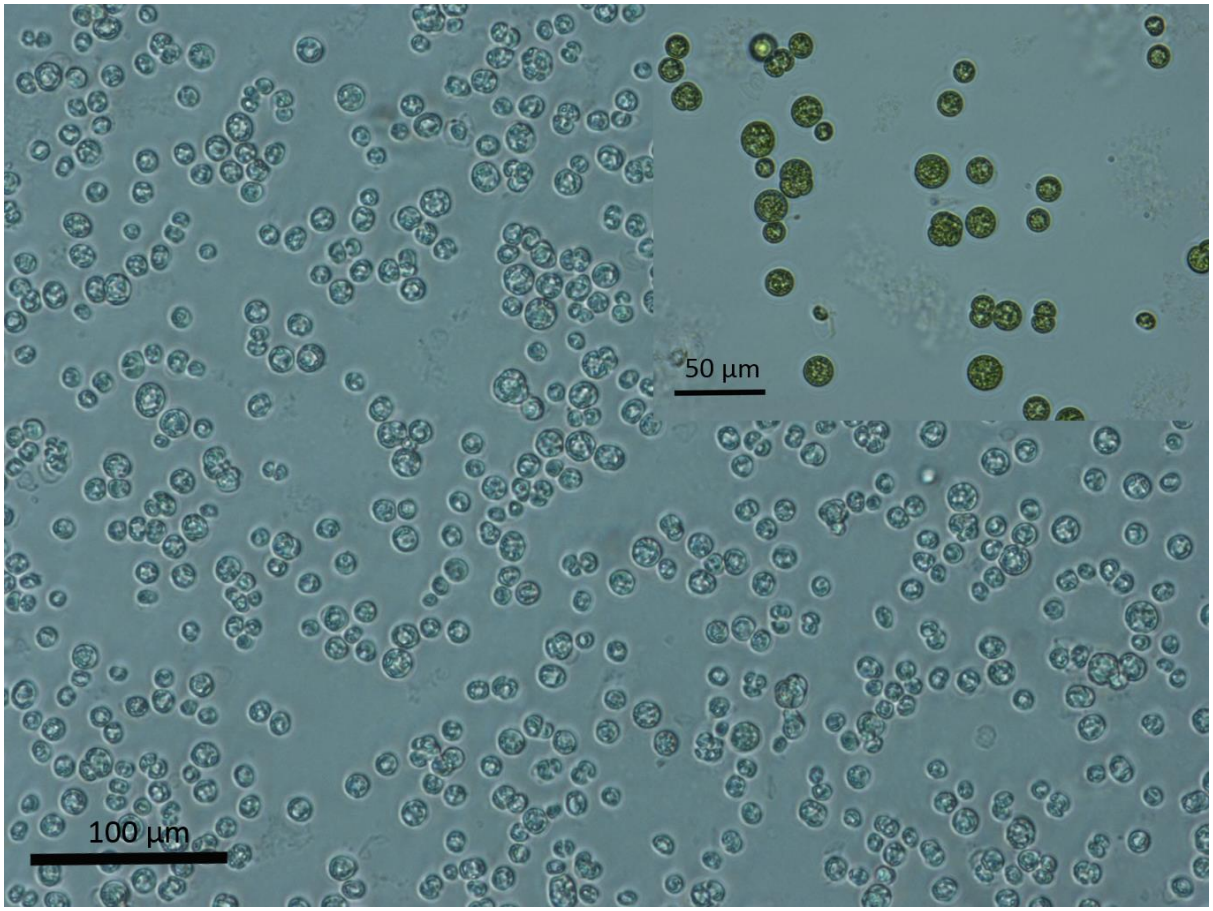

Figure 3.14. Light microscopy picture of pale mutant (main picture) of *C. emersonii* isolated by UV mutagenesis and wild-type *C. emersonii* (top right indent).

At this stage we were 1.5 years into a three-year project and not having obtained any mutants of *P. ellipsoidea* despite the efforts described above. The initial objective was to have a number of mutants at the end of the first year. It was obvious that if any mutants would be obtained that would most likely be achieved not earlier than the end of the second year of this project, therefore a decision had to be made as to the way forwards. The decision was to not carry on with mutagenesis but rather to focus on understanding the effect of the presence of algaenan in the industrial potential of certain green algal strains. The discussion that follows aims to discuss the results but also to justify the decision that was made to discontinue mutagenesis and take a different way forwards.

3.3 Discussion.

3.3.1 Challenges in developing a rigorous mutagenesis and screening protocol for the isolation of cell wall mutants in P.ellispoidea.

We used UV radiation and EMS to induce random mutations in early exponential stage *P. ellipsoidea* and high-throughput screening methods in order to isolate light-harvesting (pale) mutants as well as cell wall deficient mutants in the hope of inhibiting algaenan production. The methods used here are very similar to methods that had been successfully used before in our lab (Smith-Baedorf, 2012) and others (Huesemann *et al.*, 2009; Meireles *et al.*, 2003). Two strains, termed Ni and Obi were tested and strain Ni was chosen for subsequent experiments on the basis of better productivity, larger cell size and better lipid accumulation. As described above, developing a satisfactory protocol for the random mutagenesis of this strain proved very challenging. In particular, *P. ellipsoidea* showed a great variation in the sensitivity to mutagen depending on the growth stage. More importantly, however, a considerable amount of mutagenesis and screening efforts using UV radiation and EMS combine with high throughput screening by FACS, revealed no pigment mutants. Pigment mutants should be some of the most common and easily identifiable mutants in algal mutagenesis (Huesemann *et al.*, 2009) so their complete absence was taken as a sign that improvements had to be made to the mutagenesis protocol.

For a considerable amount of time, the reason for the absence of mutants was unclear. A review of the evidence from the mutagenesis efforts of the first 12 months suggested that one of the main possible reasons was the tendency of this species to grow in aggregates in early exponential stage. This patern of growth prevented equal dosing of the mutagen to all cells as some would be protected in the centre of aggregates, possibly leading to the persistence of wild-type cells even at high doses of mutagen. Furthermore, colonies appearing on agar plates could not be guaranteed to originate from a single cell, thus even if mutants did exist within some of those aggregates they would very likely be outgrown by the wild-type cells present in those and

give the appearance of a normal colony once they had been incubated for the three week period required for algae to show colonies on agar. A UV mutagenesis with the green alga *C. emersonii* readily yielded pigment mutants, further indicating that the challenge was species-orientated rather than method orientated.

P. ellipsoidea tends to de-aggregate at some point during the exponential growth but this is accompanied by a sharp decrease in division rates and a decrease in absolute plating efficiency (table 3.1.). Therefore, gentle sonication was tested in order to break early exponential cell aggregates and improve the mutagenesis protocol. Sonication for 20 min did generally break cell aggregates but it was later found that it also dramatically reduced the absolute plating efficiency of this strain. This in turn meant that it was not possible to get good calibration curves and to reliably determine the mutagen dose required for 90% mortality.

3.3.2 FACS screening as a high-throughput method for the isolation of pigment and cell wall mutants.

FACS screening was used after mutagenesis to provide a high throughput method of isolating putative mutants. In particular cells were sorted on the basis of high calcofluor fluorescence (putative algaenan-deficient mutant) and low chlorophyll fluorescence (putative light-harvesting mutant).

P. ellipsoidea naturally varied by more than an order of magnitude in chlorophyll fluorescence, so the 1% - 10% of cells with the lowest chlorophyll fluorescence were sorted as the population that was most likely to contain a true light-harvesting mutant. Therefore, FACS cannot be used to directly isolate mutants but rather as a method of increasing the throughput of screening by one to two orders of magnitude depending on how strict the screening criteria are.

In the same manner, calcofluor staining of wild-type cells showed that 5%-50% of cells showed some calcofluor fluorescence. This was most likely because *P. ellipsoidea* contains a polysaccharide secondary cell wall and through the process of division, gaps in the primary algaenan cell wall are present, allowing calcofluor to bind to the cell wall. Damaged or dead cells may also be stained in a similar manner (Smith-Baedorf, 2012; Zych *et al.*, 2009). Calcofluor staining was therefore used to eliminate calcofluor-negative cells and sort for calcofluor fluorescent cells. Thus, as with chlorophyll screening above, FACS should be seen as a way of increasing throughput rather than directly detecting desirable mutants. *P. ellipsoidea* was prone to loss of plating efficiency, a phenomenon not well understood at present but also reported elsewhere (Manandhar-Shrestha and Hildebrand, 2013). For that reason liquid cultures from all mutagenesis events were kept so that a backup store of mutants existed in case of loss of plating efficiency and for further rounds of FACS screening.

In conclusion, FACS seemed to be a good complementary method to increase throughput in random mutagenesis experiments. However challenges described above in developing a mutagenesis method for *P. ellipsoidea*, together with the high cost of booking time on the FACS machine (>£100/hour) meant that, unfortunately, FACS was not developed and exploited to its full potential in this study.

3.3.3 Advantages and disadvantages of modifying the P. ellipsoidea cell wall.

We were not able to obtain mutants of *P. ellipsoidea* up until the half-way mark of this project. At this point a decision had to be made as to whether it would be worth continuing to pursue random mutagenesis and how to do so. As discussed in the introduction, *P. ellipsoidea* is unique among microalgae for showing a negligible or very thin secondary cell wall (Smith-Baedorf, 2012). Although not much is known about algal cell walls and how their production is regulated, it is possible that inhibiting algaenan

production in this species would render it too weak for production of biofuels on an industrial scale. Furthermore, a number of assumptions had been made with regards to the ecophysiological significance of algaenan cell walls. The main assumption was that algaenan cell walls confer increased mechanical strength to the species producing it. Although many authors state that algaenan is a very resistant polymer that makes downstream processing difficult, nobody to our knowledge had actually tested this.

In contrast, producing an algaenan cell wall might also have benefits for the industrial culture of algae. By being non-hydrolysable and resistant to enzymatic attack, algaenan cell walls might confer increased resistance to predation/infection and limit potential predators to those that use mechanical means of breaking down their food (i.e. rotifers). It is also noteworthy that, while algaenan is not particularly common among algal species, many of the most robust species considered today for biofuel production do in fact produce algaenan cell walls (*Scenedesmus, Nannochloropsis*, some *Chlorella* spp.). This also suggests that the production of algaenan might make algal species especially robust and/or suitable for industrial culturing.

The vast majority of research on algaenan has been done from a geochemical perspective. That is research to elucidate the structure and composition, possible monomers and degradation of algaenan in geochemical timescales in order to evaluate the hypothesis that algaenan is a significant contributor to type 1 kerogens which in turn are the major source of liquid fuels today. However a literature search showed that very little rigorous and comparative research had been done to assess the ecophysiological role of algaenan and the effect of algaenan cell walls on the growth and downstream processing potential (cell disruption, lipid extraction) of industrially relevant species. Given that many of the species currently touted as potential biofuel strains produce algaenan, more research on the above would aid species selection as well as better inform life-cycle analyses of the potential variation in downstream processing costs inherent in using different species of algae.

3.3.4 Concluding remarks and the way forwards.

We were unable to obtain any pigment or cell wall mutants of *P. ellipsoidea* strain Ni despite 18 months of efforts, considerable method development and successful mutagenesis of other species. This was mainly because of the tendency of this strain to grow in aggregates in early exponential stage thus making standardisation of mutagen dosing challenging. Research on the transformation of this species by particle bombardment was published shortly after our decision to terminate our mutagenesis efforts and they used stationary stage cultures for transformation (Imamura *et al.*, 2012). In hindsight, it would have been more beneficial to continue mutagenesis for both strains provided, given that the strain which was not selected (Obi) did not show aggregates in early exponential stage. It would also have been beneficial to try mutagenising stationary stage cells. However by the time these conclusions had been drawn, the project was already at its half-way point. Furthermore a team of two postdoctoral students had worked in Chuo University (Japan) on an identical project with *P. ellipsoidea* and while they were able to isolate pigment mutants for this species, they had been unsuccessful in isolating cell wall mutants by the summer of 2012 (Hiroaki Fukuda, personal communication).

For all the above reasons it was decided that it would be more beneficial to change the focus of our investigations to understand the effect of algaenan on industrially relevant characteristics such as energy required for cell disruption, lipid extraction kinetics and the suitability of algaenan producers for hydrothermal liquefaction. This is an area where research is currently lacking, especially given the importance of the algal cell wall as an interface between the cell and the environment and as the main barrier to lipid extraction and downstream processing of algal species.

4 Growth and lipid accumulation of *Pseudochoricystis ellipsoidea* in raceway ponds and bubble column photobioreactors.

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PBRs were purchased by Aragreen and the University of Bath during a knowledge transfer project.

Jack Busby collected the data for PBRs and ponds in November/December 2012 and used a lipid staining protocol developed by Jenner-Hillard to compare lipid productivity of the ponds and PBRs under guidance and supervision of the author as part of an undergraduate research project. Figure 4.10 was adapted from his thesis (Busby, 2013).

Jane Lapworth developed the harvesting protocol as part of her undergraduate research project (Lapworth, 2012) **under guidance and supervision of the author and helped with set up, data collection and harvesting of the March 2012 pond runs.**

Raleigh Jenner-Hillard developed a semi-quantitative protocol for the detection of lipids in *P. ellipsoidea* as part of an undergraduate research project (Jenner-Hillard, 2013) **and helped with harvesting of the ponds in November/December 2012.**

4.1 Introduction.

4.1.1 Growth systems for large scale microalgal biomass production.

While a number of reviews and life-cycle analyses have shown that microalgal cultures are significantly more productive that plant-derived biofuels per unit area (Campbell *et al.*, 2010; Chisti *et al.*, 2011; Clarens *et al.*, 2011, 2010), algal biofuels are still at an immature stage of development and thus not commercially available. This is mainly a consequence of the high energy requirements for growth and downstream processing resulting in a, thus far, unfavourable energy balance (Liu *et al.*, 2013) and economics (Chisti *et al.*, 2011; Norsker *et al.*, 2010). Much research has been conducted in designing the best possible growth systems. Broadly speaking, algal cultivation systems can be divided in two categories; open systems such as

raceway ponds and closed systems that are collectively called photobioreactors (PBRs).

Open systems are fairly simple, low productivity systems with the main aim being the reduction of costs while maintaining productivity. Unstirred, earthen ponds have been used in Australia for more than two decades in a commercial operation for the production of ß-carotene from the halophilic alga *Dunaliella salina* (Borowitzka and Borowitzka, 1990; Borowitzka, 1991). While these growth systems have very low productivities, initial investment and human intervention are minimal thus also resulting in low capital costs. Raceway ponds, also called high-rate ponds, refers to a basic design of mixed pond with oval shape where a middle baffle provides a "raceway" layout for the recirculation of algal cultures and a paddlewheel usually provides stirring. These can be earthen or sealed with clay or PVC lining. This system was developed by Oswald and colleagues (Oswald and Golueke, 1960) for the treatment of municipal wastewaters and while it has not been successfully used for that purpose, raceway ponds have been used for decades in commercial algal operations for the production of food and other non-fuel products (Spolaore *et al.*, 2006) and most companies currently attempting to produce algal biofuels use raceway ponds as their cultivation apparatus (Campbell *et al.*, 2010; Craggs *et al.*, 2012; Jorquera *et al.*, 2010; Liu *et al.*, 2013). One of the main limiting factors to growing algae in open systems is that by being open to the elements they are prone to contamination and infection/predation. Therefore only extremophilic algae such as the extreme halophile *D.salina* or the few naturally dominant species (eg. *Chlorella* spp. and *Scenedesmus* spp.) can be reliably grown (Bull and Collins, 2012; Wang et al., 2013).

On the other hand photobioreactors (PBRs) generally aim to maximise algal biomass and lipid productivity, biomass density, land productivity and light conversion efficiency while maintaining costs as low as possible. A number of different systems have been invented and tested for their utility in large-scale microalgal production and this field of research is still fast-moving

as biotechnologists, engineers and inventors are trying to find an optimum design for high productivity and reliability as well as low running costs. PBRs can generally be in the form of transparent tubes, plastic bags or flat plates which can be orientated horizontally or vertically (Chen *et al.*, 2011; Cuaresma *et al.*, 2011; Degen, 2001). Vertical PBRs are also called bubble column PBRs because mixing is provided by bubbling CO_2-enriched air at the bottom of the reactor. These are generally limited by height as this increases the pressure required to bubble gas at the bottom leading to increasing costs (Hulatt and Thomas, 2011). Vertical systems have generally the highest productivities per land area and the most efficient light utilisation (Cuaresma *et al.*, 2011) although this depends somewhat on the actual layout of the PBRs as horizontal tubes can be stacked vertically on top of each other. Horizontal systems are generally mixed by pumping the culture through the reactor and have the highest specific growth rates and biomass density at harvest. A high biomass density at harvest is desirable because it decreases the harvesting costs per unit of oil produced. Because of their horizontal orientation, these systems can have poorer gas transfer rates, leading to the build-up of excess oxygen produced during photosynthesis (Chen *et al.*, 2011; Cuaresma *et al.*, 2011). Both systems have higher capital costs due to the larger number of parts and higher operational costs associated with the energetic requirements of CO_2 delivery and/or mixing. PBRs, being closed systems, are generally less prone to contamination than open ponds which are fully exposed to the elements. However they have the disadvantage of being difficult and labour intensive to fully clean if contamination does occur. PBR systems can also suffer from biofouling on the walls of the reactor thereby severely limiting the light available to the rest of the culture and significantly reducing growth rates (Arbib *et al.*, 2013).

Life cycle analyses have shown that for a low cost product such as biofuel, the use of PBRs cannot be justified in most occasions (Jorquera *et al.*, 2010), that is the increase in productivity is not enough to offset the increased running costs and reduced reliability of the system. At the moment these lines are written the paradigm in the algal R&D community is shifting

towards the use of raceway ponds for biofuel production and the development of bioreactors for high-value products such as neutraceuticals, cosmetics and pharmaceuticals. To my knowledge the only currently commercially viable PBR operations focus on producing astaxanthin, a high-value carotenoid, from *Haematococcus pluvialis* (Spolaore *et al.*, 2006) and ß-carotene from *D.salina* (Borowitzka and Borowitzka, 1990; Borowitzka, 1991).

4.1.2 Harvesting systems and challenges.

Dewatering presents a challenge unique to microalgae among biofuel crops. This is because algae are present in a dilute culture with water contents typically between 99.6% and 99.98% in PBRs and raceway ponds respectively (Chen *et al.*, 2011). Dewatering is one of the main energetic costs to algal downstream processing and much research is still underway on finding an economic and scalable process to do this.

The methods for algal dewatering can generally be divided into the following: autoflocculation (gravity settling) (González-Fernández and Ballesteros, 2012; Sukenik and Shelef, 1984), polymer flocculation or flotation (Henderson *et al.*, 2009), centrifugation and filtering (Sim *et al.*, 1988; Uduman *et al.*, 2010). Recently also sonication/ultrasonication (Bosma *et al.*, 2002) and electrocoagulation (Chen *et al.*, 2011) have been put forward as potential methods for harvesting microalgae. Dewatering usually consists of multiple steps and the extent to which water has to be removed depends on the downstream fate of the biomass. Using algae for anaerobic digestion or hydrothermal liquefaction requires a solid content of 3-10% (Ward *et al.*, 2014) which can be generally achieved using any of the methods available. Using biomass for lipid extraction means that most of the water has to be removed (Cooney *et al.*, 2009) (although the extent is still debated) by centrifugation, perhaps followed by drying.

Flocculation/flotation methods are generally reliable and well characterised but require a polymer flocculant which cannot be recycled and

the price of which will eventually impact the economics of the process (Uduman *et al.*, 2010). Some algae can flocculate without the need for additives and these certainly are attractive but autoflocculation takes longer and is somewhat dependent on the physiological state of the algae, therefore not as reliable (González-Fernández and Ballesteros, 2012).

Centrifugation is a reliable method to produce algal paste but its high operating costs mean that only pre-concentrated biomass can be treated in this way. Research is still underway on lowering the energy requirements of centrifugation but it is mainly a complementary method for dewatering (Grima *et al.*, 2003).

Filtration systems such as membrane filters can harvest algae very efficiently, economically and produce effluent of high quality but they will eventually clog and need replacement and their performance depends on cell size with larger cells being generally more suitable to filtration due to reduced clogging (Sim *et al.*, 1988).

4.1.3 Scope of this chapter.

In this chapter the set-up of growth facilities to culture *P. ellipsoidea* from lab scale to the 1 m^3 scale at the University of Bath are described. The purpose of these facilities was two-fold. Firstly to evaluate the growth of *P. ellipsoidea* in realistic conditions, to compare it to strains in the published literature and to any mutant strains developed and secondly to provide enough biomass to a collaborating student in the Department of Chemistry (Dan Adams) to carry out experiments on the transesterification of lipids to biodiesel using novel catalysts. Raceway ponds were chosen as the main culturing system as this is the same system our sponsors use in large scale culturing of *P. ellipsoidea* in Japan.

4.2 Methods and Results.

4.2.1 Growth room and PBR setup.

A temperature controlled plant growth room was converted for the purpose of growing large numbers and volumes of algae for various experiments and as seed cultures for the raceway ponds. This featured a row of fluorescent tubes to provide light, shelves with magnetic stirrers and shakers as well as a row of 14 bubble column PBRs. Light levels were 80 (± 20) μmol photons m^{-2} s^{-1} PAR for most growing positions, apart from the top shelf which had 250 μmol photons m^{-2} s^{-1} PAR. CO_2 was provided by mixing pure CO_2 with air at 2.5% v/v and distributed around the room by silicone pipes connected to manifolds. Flow meters were installed to control gas flow to the PBRs but not the shelves with flasks. Because CO_2 could not be standardised on that side, we could not have true replicates of growing flasks bubbled with CO_2 so experimental flasks were generally not bubbled.

Typical growth curves of *P. ellipsoidea* for all the different containers and positions were obtained in order to assess how growth rates and productivity varied. In particular *P. ellipsoidea* was grown in 250 ml conical flasks with 100 ml of culture in orbital shakers under 250 μmol photons m^{-2} s^{-1} PAR with no gas addition, in 1 L Duran bottles, magnetically stirred with addition of CO_2 at 250 μmol photons m^{-2} s^{-1} PAR, in 8 L PBRs with CO_2 addition under 80 μmol photons m^{-2} s^{-1} PAR in two different media; BBM which was our standard media for growth and maintenance of algal species and 4DPM, a fourfold-concentrated version of DENSO's minimal raceway pond medium.

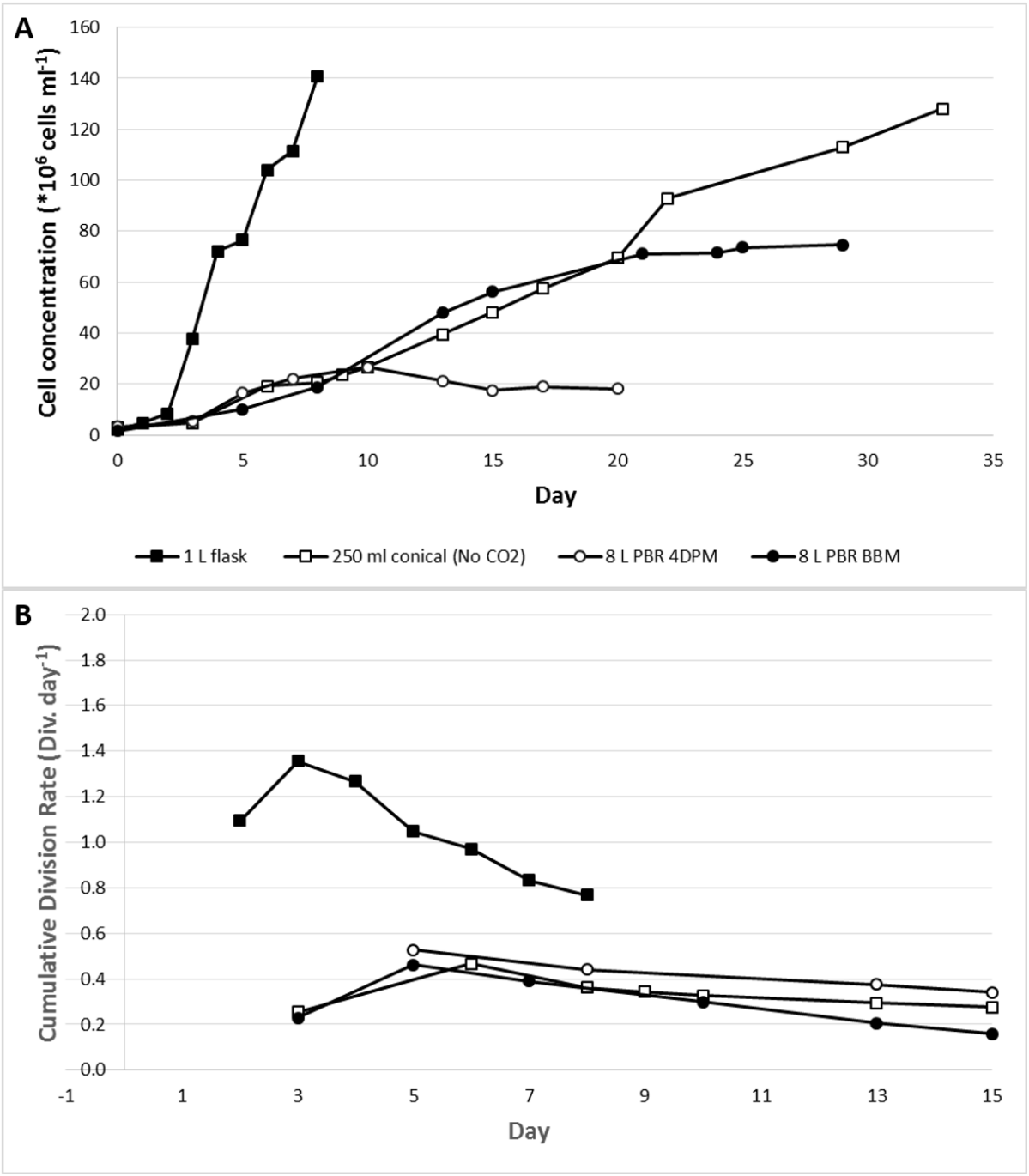

Figure 4.1. The growth of *P. ellipsoidea* in different culture vessels expressed as cell concentration (A) and cumulative division rates (calculated from the first day of exponential growth) (B). Lines represent 250 ml conical flasks with no CO_2 (open squares)(n=1), mean of two 1 L Duran bottles bubbled w/ CO_2 under high light (closed squares)(n=2), mean of three PBRs with BBM media (closed circles)(n=3), and mean of three PBRs with 4DPM media (open circles)(n=3).

Maximum cell density was $128*10^6$ cells ml^{-1} in 250 ml flasks, $141*10^6$ cells ml^{-1} in in 1 L flasks, $74*10^6$ cells ml^{-1} in PBRs with BBM media and $26*10^6$ cells ml^{-1} in PBRs with 4DPM media (fig. 4.1.A). This suggests that

our PBRs were light-limited as they reached only about half the cell density of the flasks and that PBRs with 4DPM were limited by nutrient availability as 4DPM is more dilute than BBM. Maximum measured division rates were 0.68 div day^{-1} in 250 ml flasks, 0.53 div day^{-1} in BBM PBRs, 0.81 div day^{-1} in 4DPM PBRs and 1.77 div day^{-1} in 1 L flasks (fig 4.1.B). The great difference in division rates between 1 L flasks and all other treatments suggests that the combination of high light and high CO_2 can greatly accelerate the growth of this species. It is also noteworthy that flasks with high light and no CO_2 addition showed similar growth rates and higher cell concentrations than PBRs bubbled with CO_2 and this suggests that addition of CO_2 increases growth rates as long as there is sufficient light to fix this CO_2.

4.2.2 Pond conditions and management.

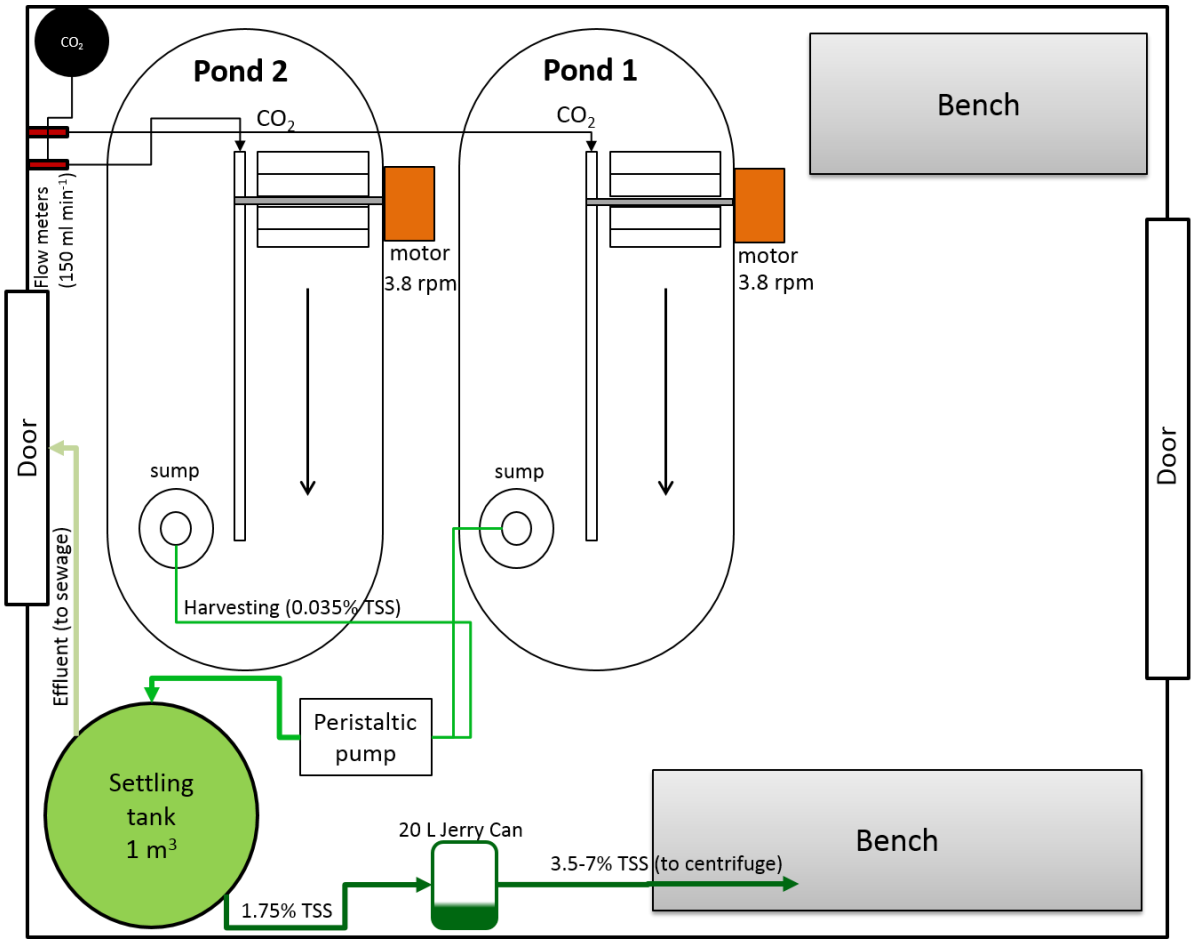

Figure 4.2. Overview of the raceway pond glasshouse layout at the University of Bath.

Two 500 L raceway ponds were constructed and set up in a glasshouse at the University of Bath (fig. 4.2.). Algal cultures growing in ponds were supplemented with artificial lights to regulate day length. The ponds received a minimum of 80 μmol photons m^{-2} s^{-1} PAR for 16 hours per day and the proportion of the artificial light supplementation to reach this photon density varied seasonally (i.e. more artificial light was appropriately supplemented in the winter to compensate for shorter day length). Algal cultures were mixed by a paddlewheel and 100% CO_2 was supplied through spargers upstream of the paddlewheel. The actual capacity (i.e. the volume of water to reach 15 cm depth) of the ponds was found to be 650 L as they were constructed slightly larger than the designs. To inoculate the cultures, ponds would be filled either up to 15 cm of depth (650 L) and allowed to evaporate over the period of growth or with 500 L of water (12 cm depth) and have the water replenished to the original depth before sampling. While the water was mixed by the paddlewheel, nutrients were added in 1000x concentrated stocks and then pH adjustments were made to a 100 ml sample from each pond and extrapolated to the total volume of the pond to determine the HCl dosage needed for a pH_0 of 3.5. The initial pH of the tap water was in the range of 7.5-8, strongly buffered in the around pH 5-7 and ponds normally required 200-300 ml of concentrated HCl (10.15 M) each. After pH adjustment the ponds were left overnight and this was done firstly to let the water temperature adjust and secondly because it is best to inoculate algae in the morning so they can have as long a photoperiod as possible to adjust to their new environment.

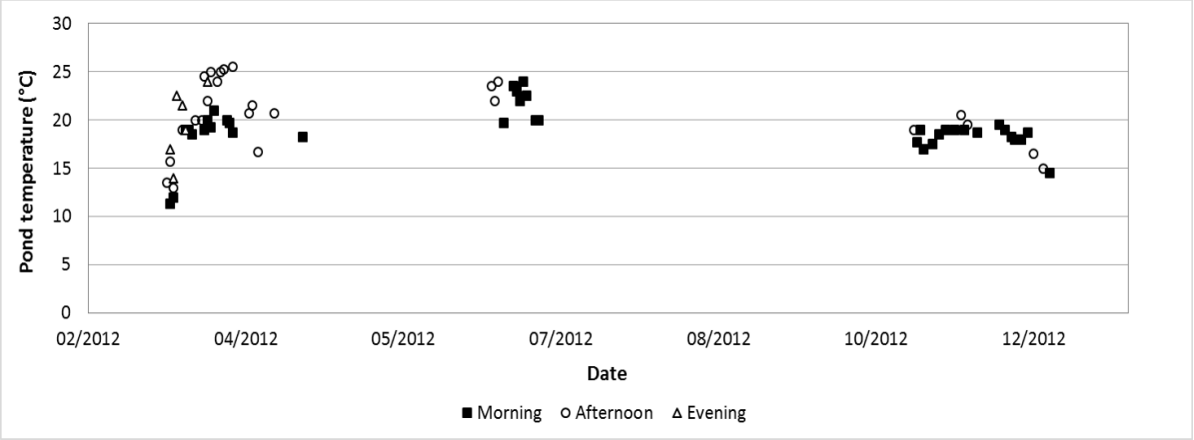

Figure 4.3. Summary of all water temperature measurents in the raceway ponds. Values are averages of the two ponds. "Morning" refers to values measure between 09:00 and 12:00, "Afternoon", between 12:01 and 15:59 and "Evening", between 16:00 and 17:00. The two ponds never varied by more than 0.5 °C.

The main runs of the ponds happened in March/April 2012 with the help of Jane Lapworth who was working under my supervision on developing the dewatering protocol for the ponds as her 3rd year project; in July 2012 with the help of Chris Ridley who was a summer student volunteer and in November/December 2012 with the help Jack Busby and Raleigh Jenner-Hillard who did their 3rd year projects under my supervision on comparing the growth of raceway ponds and PBRs and developing a method for the quantification of lipids. The help of students was required here because the nature of the operation meant that two people were needed for certain tasks, especially during harvesting. Water temperature was usually between 15 °C and 25 °C (fig. 4.3.) but because temperature sampling was generally in the morning to early afternoon the full range of temperatures experienced by the algae may not have been captured.

Before the onset of cultures, a trial was performed to determine how well *P. ellipsoidea* would compete against other fast-growing species of algae commonly handled in our lab. We inoculated 100 ml of DPM cultures in colical flasks with either *P. ellipsoidea* ($2.1*10^4$ cells ml^{-1}), a mixture of *Chlorella emersonii*, *Haematoccocus pluvialis* and a *Scenedesmus* sp. ($2.1*10^4$ cells ml^{-1}), or a mixture of *P. ellipsoidea* and the above potential contaminants ($4.2*10^4$ cells ml^{-1}). We found that out of the contaminant species only *C.*

emersonii could grow in pH 4 albeit suboptimally (fig. 4.4.). The *P. ellipsoidea*+Contaminant treatment was dominated by *P. ellipsoidea* and only few *C. emersonii* cells could be seen (<<1%).

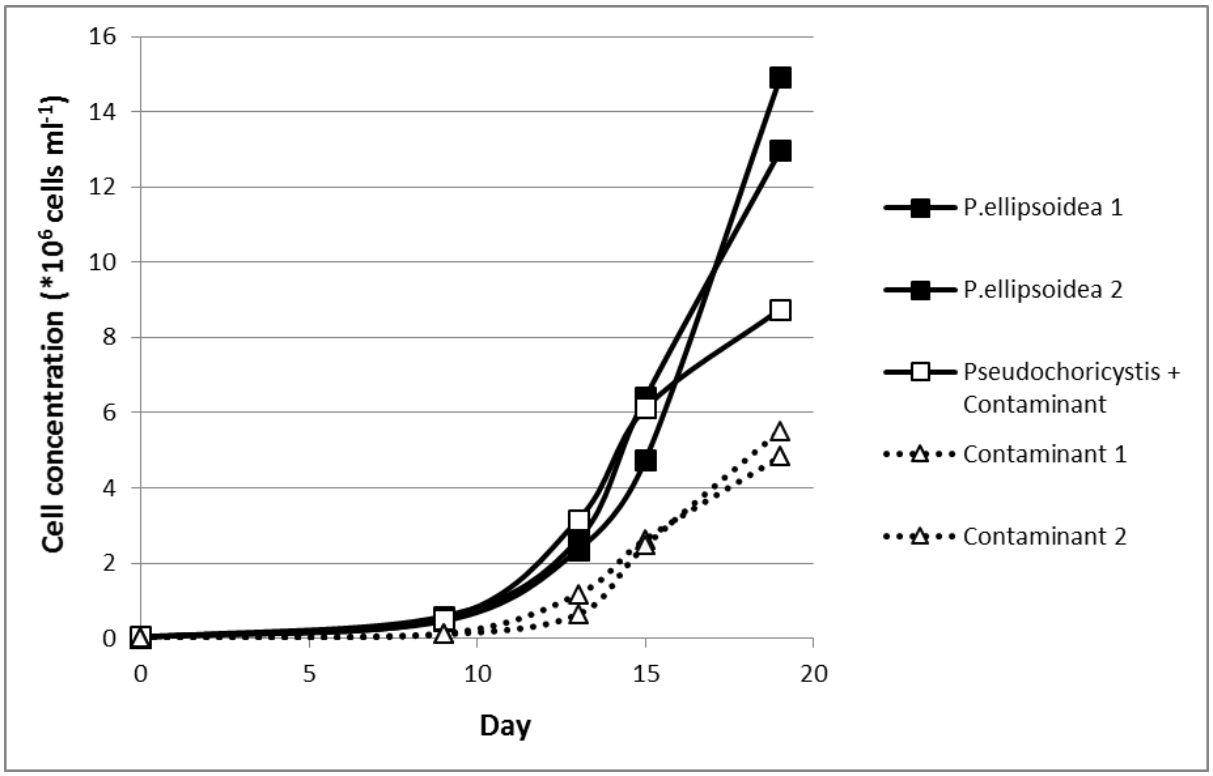

Figure 4.4. Growth monitoring of *P. ellipsoidea* and possible contaminants in DPM (pH 4) laboratory scale cultures. "Contaminant" represents a mixture of an industrial *Haematococcus plubialis* culture that had been contaminated with local wastewater community dominated by *Scenedesmus* spp. and a lab culture of *C. emersonii* Data points represent average cell count of duplicate samples from single culture flasks (n=1). *C. emersonii* dominated the two "contaminant" flasks (≥98 % of cells).

In the first run of *P. ellipsoidea* in the raceway ponds (March 2012), pH was not adjusted to test how this would affect the growth of the algae and the evolution of the pH in the ponds. There was minimal growth for the first seven days and the pH fluctuated slightly but remained between 7 and 8 (fig. 4.5.). When the pH was adjusted to pH 3 <pH_p <pH 4 on day 9, *P. ellipsoidea* showed fast growth rates. The evaporation over the 21 days of culturing was found to be approximately 220 L in each pond. Although conditions varied, if we assume steady state evaporation that would be 21 ml L^{-1} day^{-1} or 2.5 L m^{-2}

day^{-1}. Cell counts were adjusted to take this evaporation into account. The maximum growth rate measured was 1.53 divisions day^{-1} in pond 1 for days 9-12. The ponds reached a maximum average cell density of 25.4*10^{6} cells ml^{-1} (43.8*10^{6} cells ml^{-1} without adjusting for evaporation) (fig. 4.6.A) and a cumulative division rate of 0.55 divisions day^{-1} (0.59 divisions day^{-1} without adjusting for evaporation) from the onset of exponential growth till harvesting (fig 4.6.B).

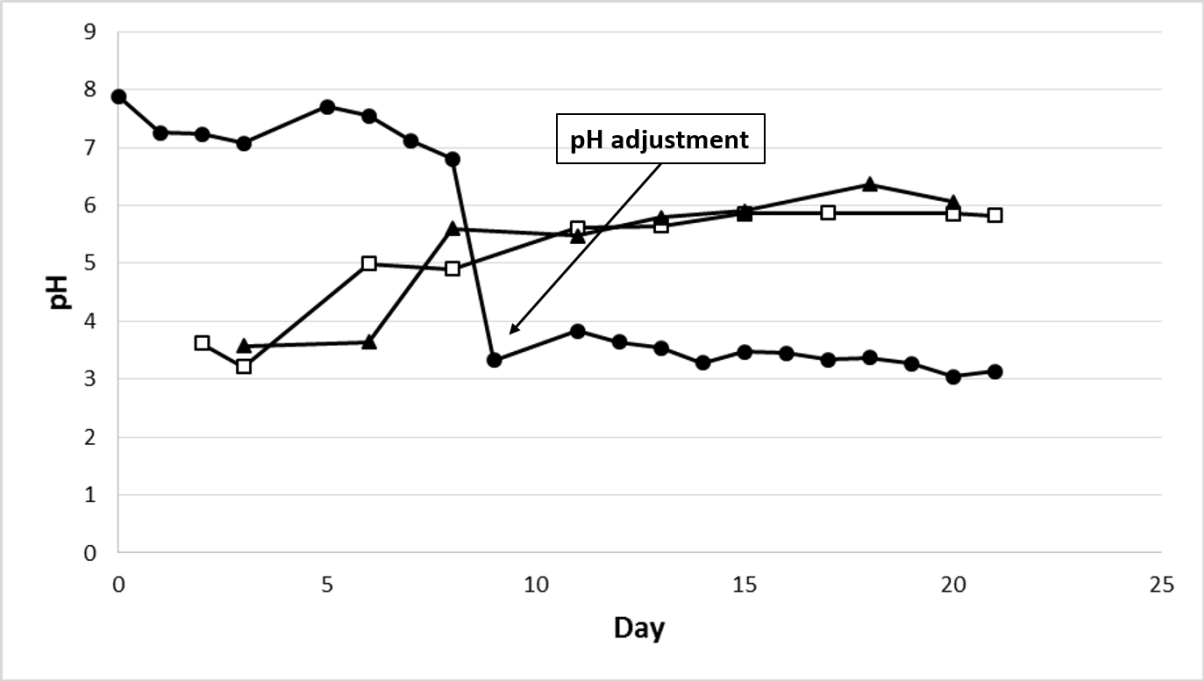

Figure 4.5. Pond pH levels in three *P. ellipsoidea* raceway pond runs. Filled circles represent the March 2012 run with pH being adjusted on day 9 and ponds being left to evaporate. Empty squares and filled triangles represent the November 2012 and December 2012 runs respectively where water was added daily to counteract evaporation resulting in a steady increase in pH. Values are averages of the two raceway pond values.

Two more pond runs took place in November and December 2012. This time the pH was adjusted to 3.6 (±0.4) and water was replaced daily before sampling up to the original water level to ensure that the total volume remained approximately 500 L. The pH of the ponds increased steadily to a final value of pH 6.1 (±0.3). Maximum cell densities were very similar to the adjusted value of the March run at 27.7*10^{6} cells ml^{-1} and 25.3*10^{6} cells ml^{-1} for the November and December runs respectively.

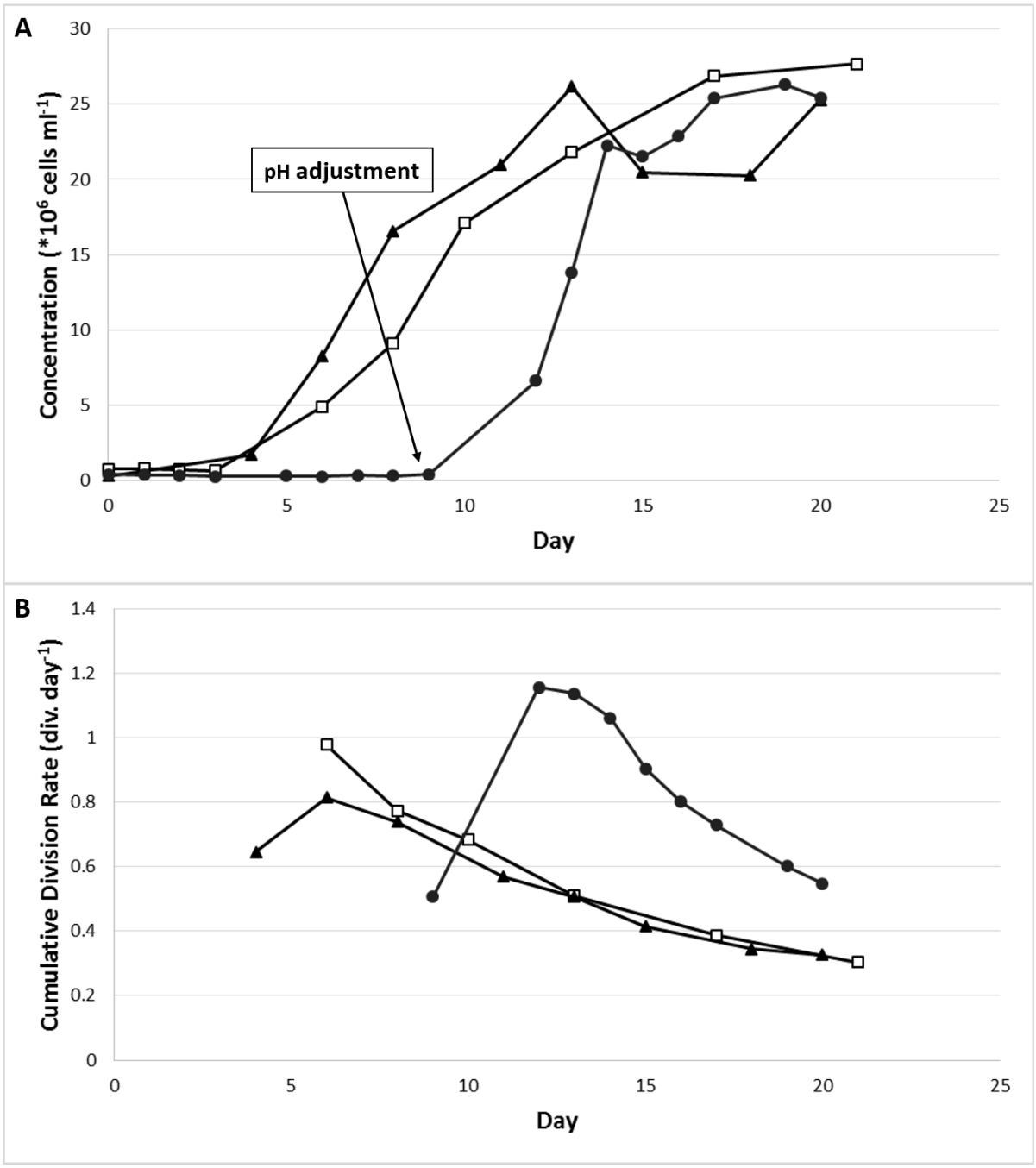

Figure 4.6. Cell concentration (A) and cumulative division rates (B) of *P. ellipsoidea* in three raceway pond runs. Filled circles represent March 2012, empty squares November 2012 and filled triangles December 2012. Values are averages of duplicate flow cytometry cell counts from each of the two raceway ponds. pH was adjusted from ~7.0 to ~3.5 on day 9 of the first run (fig. 4.5) to achieve optimal pH for *P. ellipsoidea*. In subsequent runs the pH was adjusted to ~3.6 before inoculation (fig. 4.5).

Contrary to the final cell densities, division rates were considerably lower in the last two runs with maximum division rates recorded being 1.02 divisions day^{-1} (pond 1, day 3-6) and 1.17 divisions day^{-1} (pond 1, day 4-6) for the November and December runs respectively, while in March maximum

division rates were 1.53 divisions day^{-1} (pond 1, day 9-12). The cumulative division rates from the first day of exponential growth to the final day of culturing were virtually the same throughout the growth period (fig. 4.6.B) and were measured at 0.30 divisions day^{-1} and 0.32 divisions day^{-1} for the November and December runs respectively while in March cumulative division rates were 0.55 divisions day^{-1}. The increased division rates in March may be explained by the coincidence of algal growth with a week of sunny weather in March/April 2012 and by the increased day length in March compared to November and December.

Figure 4.7. Growth and flocculation of *P. ellipsoidea* in raceway ponds (December 2012). A, after inoculation (Day 0), B, on Day 7 and C, after addition of flocculant and adjustment of pH before transferring to the settling tank (Day 21).

Harvesting was performed initially by chemical flocculation. This was done by adding 20 mg L^{-1} of chitosan in the ponds and then adjusting the pH

to pH 10< pH_p< pH 11 by addition of 10 M NaOH. The culture was then mixed gently for approximately 30 minutes and then was allowed to flocculate for approximately two hours. Initially algae were transferred in the settling tank before the addition of chitosan. Flocculation and pH adjustment in the harvesting were difficult, inefficient and potentially unsafe as it required a person to stand on a ladder and use a long stick to mix the culture as best as possible while adding chitosan and concentrated NaOH. In subsequent runs chitosan and NaOH were added in the ponds and mixed by the paddlewheel for 30 minutes. This method gave excellent results (fig. 4.7.C) giving a flocculation efficiency above 99%. However we had to ensure that the flocculated algae were pumped particularly gently to the bottom of the harvesting tank in order to keep the biomass flocs intact. After settling the algae for two hours the flocculated culture was transferred from the bottom of the harvesting tank to a 20 L jerry can and allowed to settle further (fig. 4.8.). The supernatant was gently pumped out leaving us with 5-10 L of concentrated algal biomass and giving a concentration factor of 100x-200x.

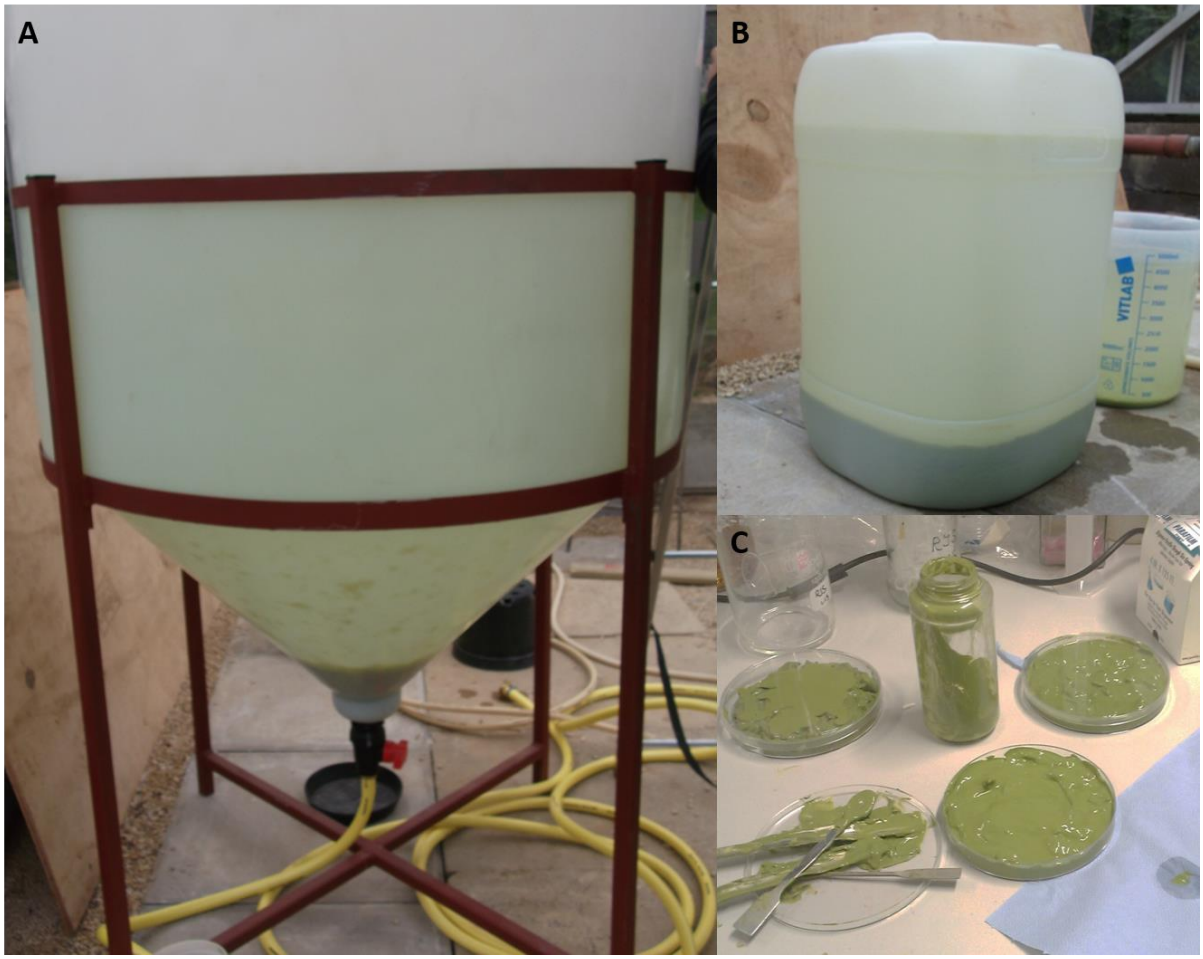

Figure 4.8. Harvesting system for *P. ellipsoidea*: A, conical harvesting tank with flocculated biomass visible at the bottom, B, further concentration of biomass overnight in 20 L jerry cans and C, wet algal paste after centrifugation.

The next dewatering step was to centrifuge the concentrated biomass and store the algal paste in petri dishes at -80 °C until freeze-drying. The ponds and paddlewheels were cleaned by hand using a 1% v/v bleach solution and washed with water twice before being re-inoculated or allowed to stand.

4.2.3 *Comparing productivity and lipid accumulation in raceway ponds and PBRs.*

Lipid content of the algal cells is an important factor in biofuel production as most of the final product (i.e. biofuel) will come from the neutral lipid fraction of the algal cells. Quantification of intracellular lipids is

not a straightforward process and generally requires an extraction procedure which, apart from time-consuming, can be unreliable depending on the extraction method and the algal cell wall (Chen *et al.*, 2010). A semi-quantitative, flow cytometric protocol for the staining of intracellular lipids using the neutral lipid stain BODIPY$^{493/503}$ was developed under the author's supervision during the course of the testing *P. ellipsoidea* growth in different conditions and reactors (Jenner-Hillard, 2013). This enabled us to compare productivity and lipid accumulation in raceway ponds and PBRs. The experiments described here took part in December 2012 and the ponds and PBRs were grown synchronously. Ponds were grown in DPM media while PBRs were grown in 4DPM media.

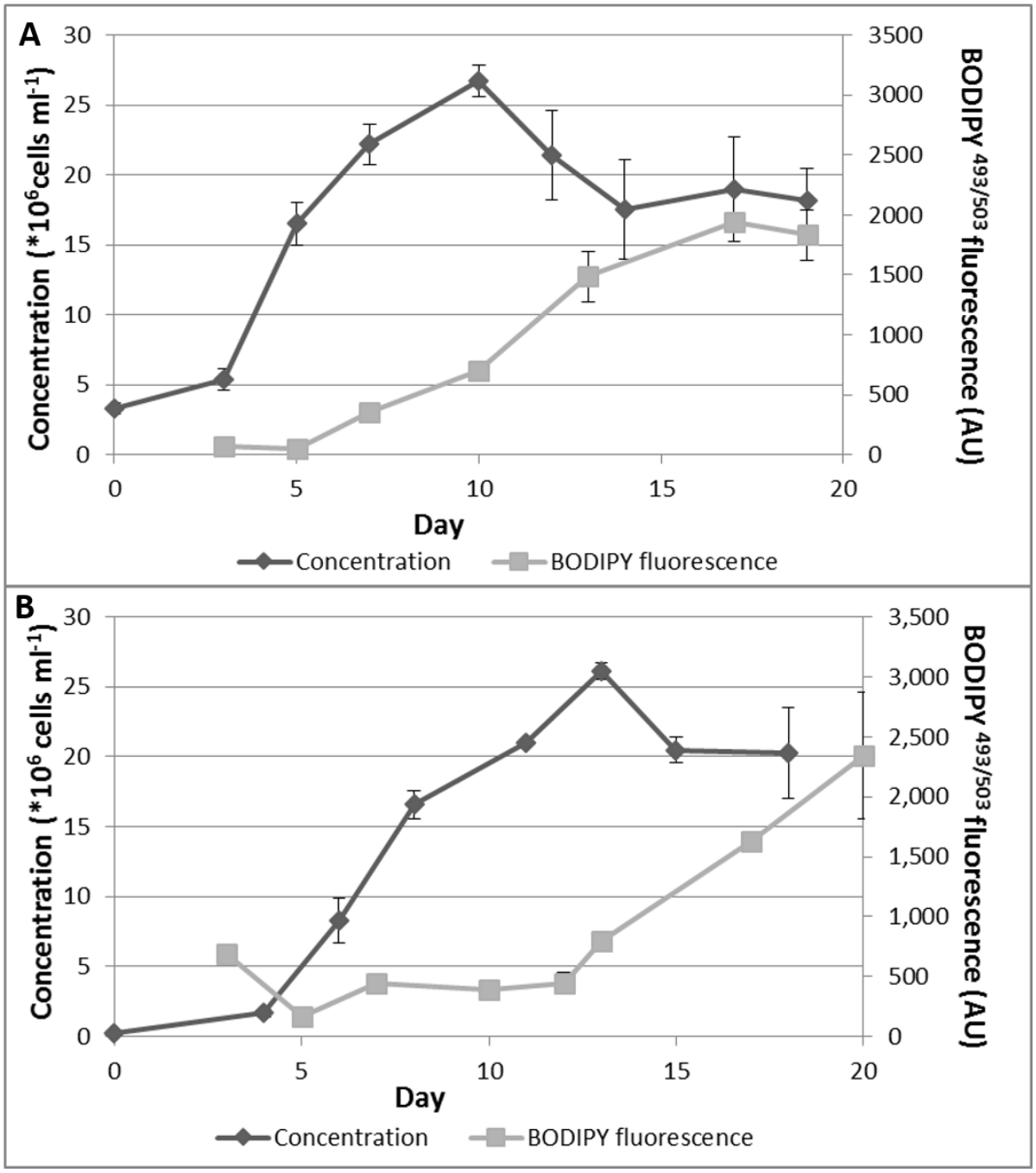

Figure 4.9. Cell concentration (dark grey) and lipid fluorescence (light grey) of *P. ellipsoidea* in photobioreactors (n=3) (A), and raceway ponds (n=2) (B) (November/December 2012). Error bars represent ±1 SE.

Both PBRs and ponds reached a maximum cell density of ~26*10^6 cells ml^{-1} (fig. 4.9.). This was somewhat surprising given that the media in the PBRs was 4x concentrated, the temperatures more stable and the light path and light levels similar to the ponds. This suggests that the PBRs were light-limited so that the higher levels of nutrient could not be fully utilised for the

production of biomass. BODIPY fluorescence increased from day 5 to day 17 in PBRs and from day 12 to day 20 in ponds (fig. 4.9.). While BODIPY fluorescence had peaked in the PBRs by day 17, in ponds it was still increasing at the last day of growth. Maximum BODIPY fluorescence per cell was 2437 AU in ponds and 1952 AU in PBRs.

Biomass concentration peaked at 0.61 g L^{-1} in PBRs (day 12) and 0.38 g L^{-1} in ponds (day 18) (fig. 4.10.). Maximum measured biomass productivity in ponds was 0.0417g L^{-1} day^{-1} (day 6 – day 8) while in PBRs it was 0.0666 g L^{-1} day^{-1} (day 3 – day 5). The fact that biomass content in PBRs was considerably higher than ponds while cell density was not, suggests that cells in PBR cultures were larger and/or heavier than in ponds. This in turn suggests that the difference in lipid content on a %dw basis would be larger than the difference in lipid fluorescence on a per cell basis. Lipid in pond cultures was quantified by chloroform/methanol sequential room temperature extraction to be 30.1% dw while unfortunately lipid in the PBR cultures was not quantified.

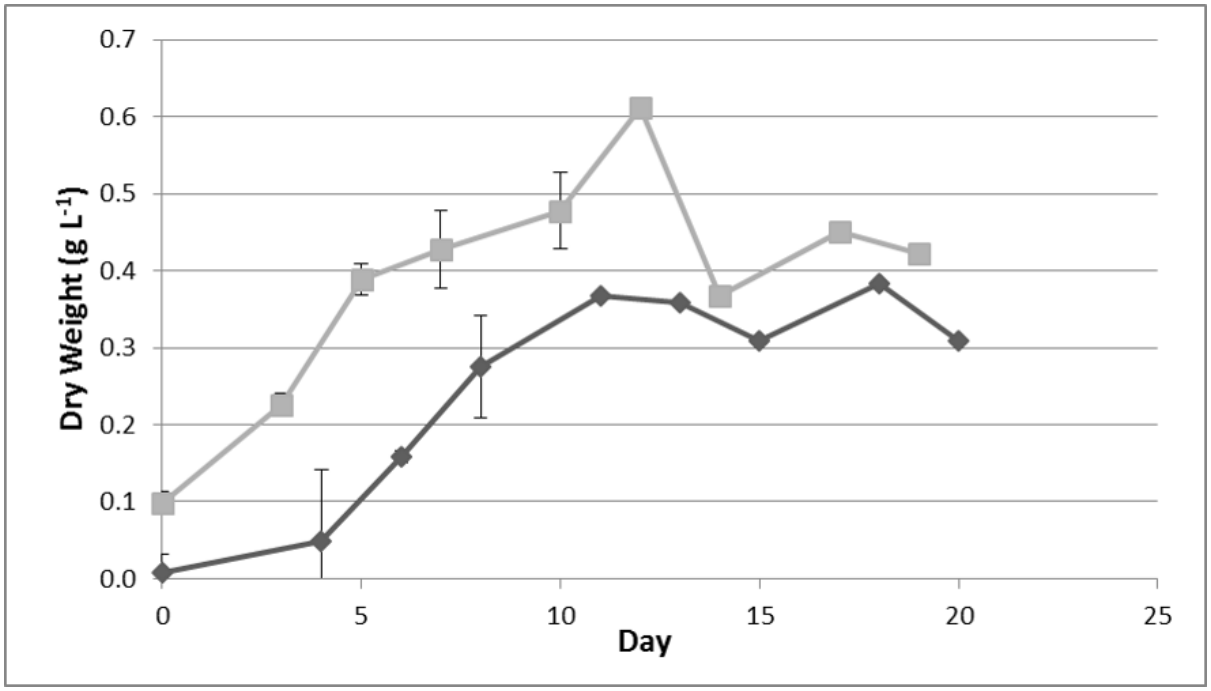

Figure 4.10. Biomass concentration (DW) in photobioreactors (n=3) (light grey) and raceway ponds (n=2) (dark grey). Error bars represent ±1 SE.

The above results suggest that PBR cultures of *P. ellipsoidea* were suboptimal for nutrient utilisation of the biomass as well as lipid accumulation in stationary stage. They also suggest that the balance between light intensity, light path and nutrient content should be carefully considered in large scale cultures in order to maximise productivity and nutrient utilisation efficiency.

4.3 Discussion.

4.3.1 Growth room and pond system performance and conclusions.

By assessing growth and division rates in cultures of different volumes and conditions we found a strong interaction between light intensity, CO_2 supply and division rates. That is when both high light and CO_2 were supplied, division rates showed a considerable increase in the growth room as well as in the ponds, were division rates in March/April were considerably higher than those in November/December, possibly due to the longer day length and coincidence of a number of sunny days during the March/April pond culturing of *P. ellipsoidea*. On the other hand the final biomass density seemed to be more dependent on the light path and nutrient concentration of the cultures. Furthermore, growing *P. ellipsoidea* in raceway ponds and PBRs with BODIPY staining to reveal lipid content showed that there is a relationship between nutrient depletion, light limitation and lipid accumulation in this species. All the above largely agree with the published literature on algal growth systems and optimising productivity (Richmond *et al.*, 2003; Weissman and Goebel, 1987).

In an industrial context these results suggest that to attain the best possible growth rates, the supply of CO_2 has to be analogous to the supply of light as either of these two can limit growth rates. The maximum biomass density is dependent on the supply of light (intensity and light path) as well as the nutrient concentration of the media. Maximum biomass densities in excess of 5 g L^{-1} were observed in 250 ml flasks without CO_2 supply while

none of the other culture conditions exceeded 3 g L^{-1} and this suggests that the ample supply of light and short light path (~ 2 cm) were the main reason for the high final biomass density. For lipid accumulation it is important that nutrients become deplete as this consists an important environmental trigger for the accumulation of storage lipids in the cells. If nutrients become limiting before light then biomass density is suboptimal and if light becomes limiting first then nutrients are under-utilised and the culture will experience a significant reduction in lipid productivity. It is clear to us that to optimise a system as such for maximum biomass and lipid production with minimum expenditure, significant modelling to establish the optimum relationship between inoculum size, nutrient content, light supply, light path length and CO_2 supply has to take place.

While the raceway ponds in this study showed good lipid accumulation that continued to increase into stationary stage, PBRs were probably light limited because of the 4x concentrated medium used in the study leading to a considerably lower lipid accumulation. Given that most algal species in our lab are cultured in BBM which is approximately eight times more concentrated than DPM, the addition of more lights to the growth room would be beneficial for future experiments.

4.3.2 What is the best culturing system for biofuels?

Raceway ponds and unmixed ponds are the only systems that have been used to culture algae in industrial volumes and time scales (i.e. several years) (Borowitzka and Borowitzka, 1990; Borowitzka, 1991; Spolaore *et al.*, 2006). This is because of the simplicity and scalability of these systems. At the time of writing these lines only Sapphire Energy seems to be able to produce algal biofuels on a pilot scale and even this effort yields almost no return on investment (i.e. the energy required to culture and process the algae is the same as the energy in the resulting biofuel) (Liu *et al.*, 2013). We have shown that algal growth rates for the same species can vary

dramatically depending on the precise conditions employed and especially light availability, light path (or vessel shape), nutrient concentration and CO_2 supplementation. This highly increased growth rate when conditions are optimised has been the main motive for the development of closed photobioreactors (PBRs) which come in a number of different configurations and are still a very active area of research. We have shown that a simple, low technology PBR offers little advantage over raceway ponds in terms of productivity while being a lot more expensive to construct and run. Although our costs do not exactly represent industrial requirements it is noteworthy that 112 L of PBR capacity had a similar cost to 1000 L of raceway pond capacity while also being more labour intensive to clean and set up.

Thus our work suggests that PBRs are worth pursuing as an algal culturing system only under very specific conditions. These are having a PBR design that is low maintenance and low energy while giving high growth rates as well as producing a high-value product along with biofuel in order to improve the profitability of the whole operation. Therefore the current paradigm is that raceway ponds are the culturing system of choice for the production of biofuels, feed and other low value products while PBRs can only be justified if a high-value product is produced (antioxidants, nutraceuticals, pharmaceuticals).

5 Algaenan cell walls in oleaginous microalgae and their significance in biofuel production: a six-species screen of green algae (Chlorophyta).

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Dimitrios Kaloudis conceived and designed the experiments, selected the species, obtained wastewater, carried out dilution optimisation, growth monitoring and microscopy pictures of algal species and communities, maintained, grew and dried the algae, did the ultrasonication of the algae, the cell counts of ultrasonicated algae, measured the cell wall thickness from TEM images, extracted and quantified the lipids, did the statistical analysis, interpreted the results and wrote this chapter.

Patrick Biller performed the HTL, biochemical and elemental analysis of dried algae grown by Dimitrios Kaloudis.

Yeling Tang did the preparation of samples for TEM, took light microscopy pictures of ultrasonicated algae and provided assistance with aspects of culturing and flow cytometry in the ultrasonication/TEM experiment.

Chris Chuck provided expert advice on lipid extraction from algae and contributed significantly to the experimental design of the lipid extraction experiments as well as providing the labspace and equipment for lipid extraction to take place.

5.1 Introduction.

5.1.1 Species selection and the potential significance of the algal cell wall in matching species to purpose.

Many of the most robust algae considered currently for industrial applications (eg. *Scenedesmus*, *Nannochloropsis*, and some *Chlorella* spp.), possess a peculiar primary cell-wall polymer termed algaenan (Kodner *et al.*, 2009). Algaenan is somewhat similar in composition to plant cuticles (cutin,

cutan) and the exine wall of pollen grains (sporopollenin) in that it is a very-long-chain (C18 – C80+), highly aliphatic, polyester heteropolymer (Allard *et al.*, 2002; Blokker *et al.*, 2006) (see section 1.5.2).

The extreme chemical stability of algaenan means that it is selectively preserved in sediments and has likely had a significant contribution to type I kerogens and current fossil oil deposits (Derenne *et al.*, 1992; Walters, 2006). This "bioplastic" polymer is also likely to have significant effects on the industrial utilisation of algae as it has been cited in the literature as reducing protein extractability (Safi *et al.*, 2013) and anaerobic digestibility (Mussgnug *et al.*, 2010), increasing mechanical strength (Hagen *et al.*, 2002), increasing resistance to detergents (Corre *et al.*, 1996), being non-hydrolysable by common cell-wall lytic enzymes (Atkinson *et al.*, 1972; Smith-Baedorf, 2012) and relatively impermeable to various stains (Smith-Baedorf, 2012; Zych *et al.*, 2009)

The biosynthetic pathway and ecophysiological functions of algaenan are relatively unexplored, however similar polymers in plants are generally said to act as a barrier to water loss and infection(Albersheim *et al.*, 2011b) and the same has been hypothesised for algaenan (Kodner *et al.*, 2009).

So far, screening for microalgal species has focused mainly on two characteristics; biomass productivity and lipid productivity (Griffiths and Harrison, 2009; Yang *et al.*, 2010). Given the above, cell wall composition may be an important factor to consider when matching species to downstream processes, especially when the choice is between species of similar performance. The presence of algaenan in a considerable number of strains with robust and fast growth as well as high lipid content together with the uncertainties in downstream processing and the proposed significance of the cell wall in the energy consumption and/or efficiency of these processes means it is important to elucidate the role of algaenan production in the suitability of species to downstream processes. So far, little comparative research has been done to understand the effect of algaenan production on industrially relevant characteristics such as the energy required for cell disruption and

the effect of algaenan on lipid extraction kinetics. Given the resistant nature of algaenan, differences are likely to exist between algaenan-walled and polysaccharide-walled species and these differences may impact the selection of species for industrial applications as well as the cell disruption and/or lipid extraction burden in life-cycle analyses.

5.1.2 Scope of this study.

The purpose of this study is to elucidate the role of algaenan production among Chlorophyta pertaining to the suitability of species to certain challenging up- and down- stream processes that are currently the object of intensive research as described above. Our approach was to screen six species of fast-growing, oleaginous green algae. First we considered the two main sources of "waste" nutrients; anaerobic digestate (AD) and municipal secondary treated wastewater (WW) and assessed the suitability of our screen to growing in these. Then we considered suitability to cell disruption and solvent extraction (the biorefinery approach) and conversion of the whole biomass to biocrude oil by HTL. We assessed six species of green microalgae, three of which were known to contain algaenan. Our strains were selected for their high growth rates, lipid yields and robustness in industrial cultures, based on the literature and our own experience. We assessed; cell disruption by ultrasonication at late exponential and stationary phase, lipid extraction kinetics by sequential solvent extraction with chloroform/methanol (2/1 v/v) at room temperature and hydrothermal liquefaction at 350 °C for 15 minutes.

Apart from assessing how cell wall variability and especially algaenan production affect the downstream processing requirements for different species of green microalgae, we also discuss our findings in terms of the ecophysiological function of algaenan cell walls in green microalgae.

5.2 Results.

5.2.1 *Species selection.*

The microalgal strains *Chlorella emersonii* CCAP 211/8p, *Chlorella* FC2-IITG, *Chlorella vulgaris* CCAP 211/11b, *Chlorella vulgaris "minutissima"* CCAP 211/52, *Chlorella zofingiensis* CCAP 211/51 and *Pseudochoricystis ellipsoidea* were selected for their high growth rates, high lipid contents and robustness in industrial culture conditions as revealed by the available scientific literature; that is these strains all have good potential to be utilised as industrial biofuel producers in the future (Bhatnagar and Bhatnagar, 2010; Griffiths and Harrison, 2009; Makarevi *et al.*, 2011; Satoh *et al.*, 2010; Tang *et al.*, 2011). Furthermore these strains were selected for their varying ability to produce algaenan cell walls with *C. emersonii*, *C. minutissima* and *P. ellipsoidea* having the proven ability to produce algaenan cell walls (Kodner *et al.*, 2009; Smith-Baedorf, 2012). *C. vulgaris* has been proven to not produce algaenan (Smith-Baedorf, 2012) and for the other two strains there is currently no data on algaenan production but on the basis of preliminary experiments with calcofluor and other stains we assume that these species do not produce algaenan. We therefore devised an experimental programme to assess the effect of producing algaenan in potential industrial situations such as growth in non-sterile anaerobic digestate and wastewater, cell disruption by ultrasonication, solvent extraction of lipids and hydrothermal liquefaction in closely related species of oleaginous green algae.

5.2.2 *Culturing in anaerobic digestate medium (ADM).*

OD_{550} measurements were used to compare biomass accumulation between species as this wavelength corresponds to minimum absorption by chlorophyll and is a good indicator of biomass accumulation within the green algae. All six species were able to grow in autoclaved and non-autoclaved anaerobic digestion medium (ADM). *C. minutissima*, *Chlorella* FC2 IITG and *C. zofingiensis* showed the highest OD_{550} in both autoclaved and non-

autoclaved ADM (fig. 5.1.). In all cases non-autoclaved OD was lower than autoclaved with the notable exception of *C. zofingiensis* which had an OD of 2.702 and 2.733 in the autoclaved and non-autoclaved ADM respectively. It is also noteworthy that the OD was still increasing between 21 and 26 days when the last two samples were taken. This implies that none of the six species tested reached their maximum biomass content for this growth medium.

Viable cell counts were used to compare growth within species, between treatments (fig. 5.2.) as OD measurements may be inflated in the non-autoclaved ADM by bacterial growth. Viable cell counts were higher for the autoclaved treatment in all species with the exception of *C. zofingiensis* were cell counts for the non-autoclaved treatment were higher than the autoclaved ADM on days 22 and 26 albeit not significantly ($p>0.05$).

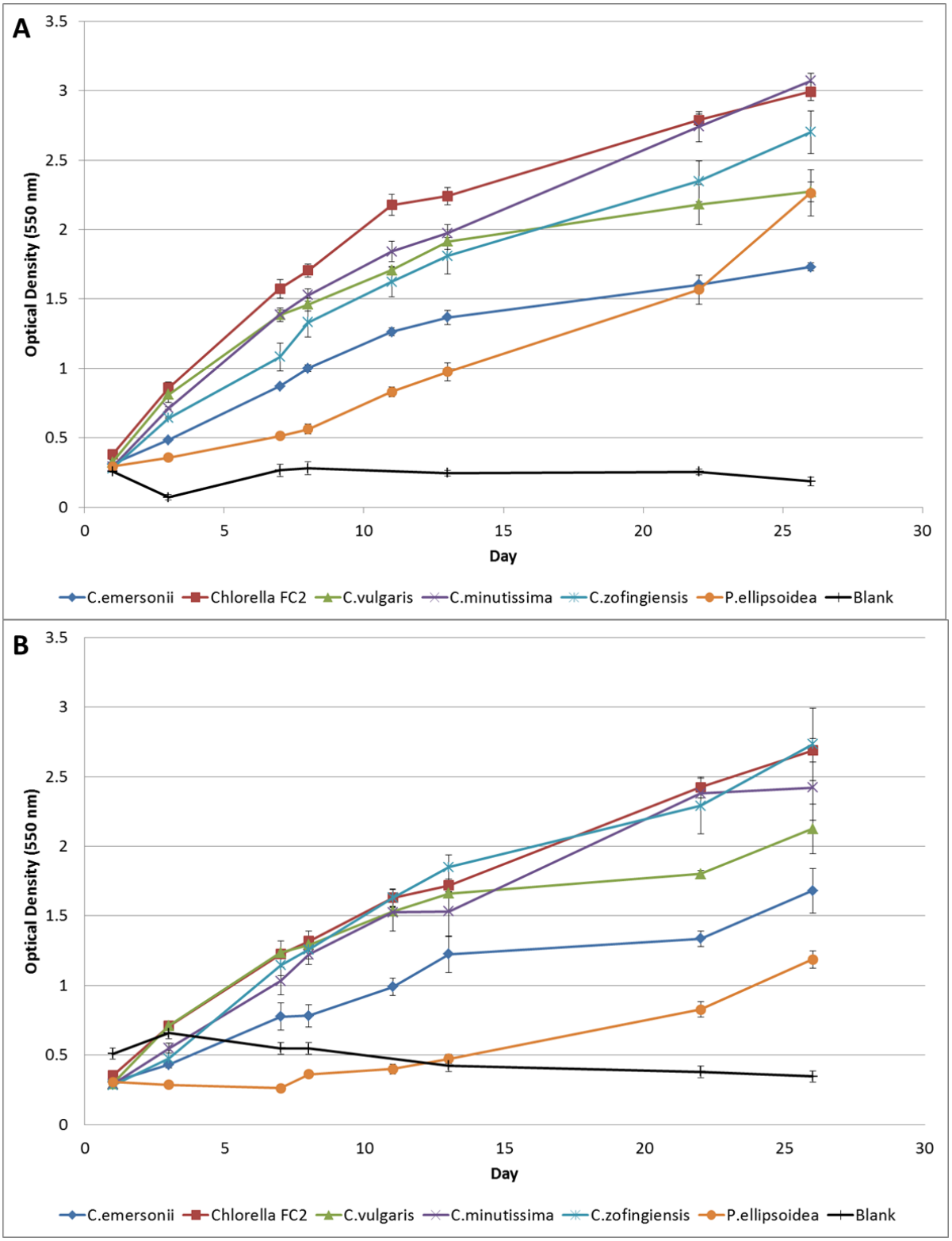

Figure 5.1. Optical density at 550 nm of six species of microalgae and blank (no addition of algae) in: A, sterile (autoclaved) and B, non-autoclaved anaerobic digestate (0.4% vol/vol), n=3, error bars represent ± 1 SE.

The species that was most affected by the sterility of the medium was *P. ellipsoidea* which had approximately half the OD_{550} (1.187 and 2.263 in non-autoclaved and autoclaved ADM respectively) and 39.2 % of the cell counts ($2.17*10^6$ cells ml^{-1} and $5.53*10^6$ cells ml^{-1} at day 21 in non-autoclaved and autoclaved ADM respectively). Our data suggests that the growth of most species was somewhat affected by the sterility of the ADM with the exceptions of *C. zofingiensis* which was not affected and *P. ellipsoidea* where growth and biomass accumulation were severely reduced in the non-autoclaved treatment. Furthermore our data shows that all six species in our screen were able to grow in diluted anaerobic digestate independently of its state of sterility. This suggests that anaerobic digestate is a good alternative source of "free" nutrients for algal growth. On day 26 the viable cell counts of the species *C. emersonii*, *C. zofingiensis* and *P. ellipsoidea* were significantly lower than day 21 in both autoclaved and non-autoclaved ADM while OD measurements showed that biomass accumulation in these treatments had increased. We speculate that this reduced cell count is not due to a loss in viability but rather due to the tendency of these species to form cell aggregates under certain conditions. This would result in an artificially low cell count as small cell aggregates would be able to pass through the flow capillary and thus would be counted as one cell in the flow cytometer. Therefore day 26 cell counts were ignored when fitting exponential growth models to the curves and in the subsequent statistical analysis.

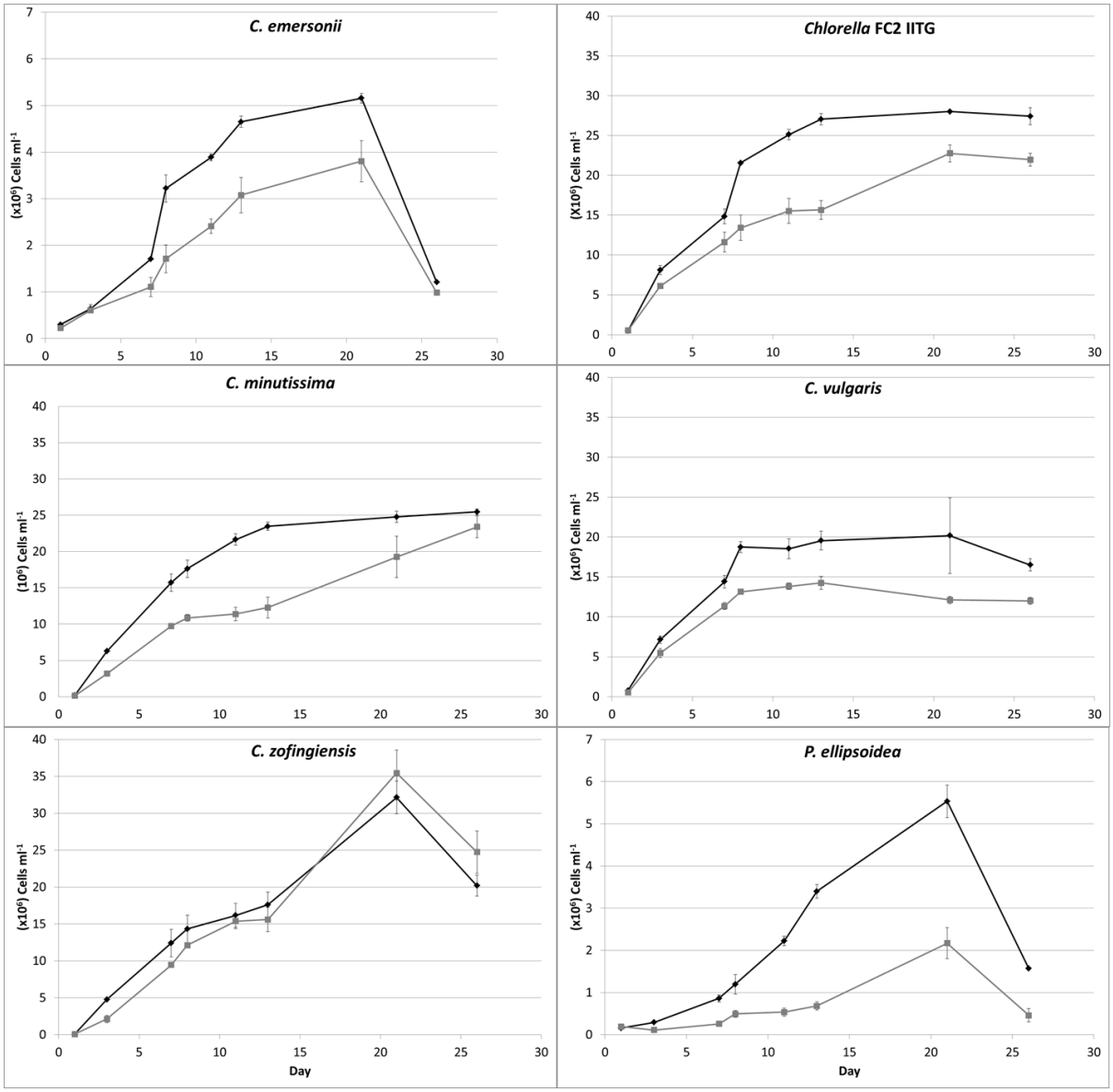

Figure 5.2. Cell counts by flow cytometry of viable algal cells in autoclaved (black line) and non-autoclaved (grey line) anaerobic digestate (0.4% vol/vol) for six species of microalgae. Error bars represent ± 1 SE (n=3).

5.3.1 Culturing in municipal wastewater final effluent.

As in the previous section OD_{550} measurements and viable cell counts were used to compare between species within treatments and within species between treatments respectively. All species were able to grow in autoclaved WW (fig. 5.3.A), with *Chlorella* FC2 IITG, *C. minutissima* and *C. zofingiensis* showing the highest OD_{550} values throughout the growth period. *C. emersonii* and *P. ellipsoidea* showed the lowest OD_{550} values for most of the experiment

but their OD_{550} continued to increase throughout the experiment and both of these species had surpassed *C. vulgaris* on the final day (day 21).

In non-autoclaved WW (fig. 5.3.B), a rapid increase OD_{550} was observed in *Chlorella* FC2 IITG, *C. minutissima* and *C. zofingiensis* until days 6-8. A decrease in OD_{550} followed the rapid period of growth and by the end of the experiment all of the above species had an average optical density lower than that of the "blank" treatment (negative control where the native community allowed to grow with no algae added). The highest OD_{550} values by day 21 were seen in the flasks inoculated with *C. vulgaris* and *C. emersonii* followed by the blank (no algae added) treatment. Cell counts by flow cytometry, however, revealed that *C. vulgaris* was almost completely absent from the non-autoclaved WW by day 8 and did not recover until the end of the experiment (fig. 5.4.). Light microscopy imaging revealed that two out of three *C. vulgaris* flasks had been dominated by a native cyanobacterium (Appendix 8.2.). This resulted in *C. vulgaris* having a higher average OD_{550} in the non-autoclaved media (0.805 and 0.584 in non-autoclaved and autoclaved media respectively). This shift to a cyanobacterial dominance was not seen in any of the other non-autoclaved flasks and even the blank treatment was dominated by eukaryotic green algae rather than cyanobacteria (Appendix 8.2.).

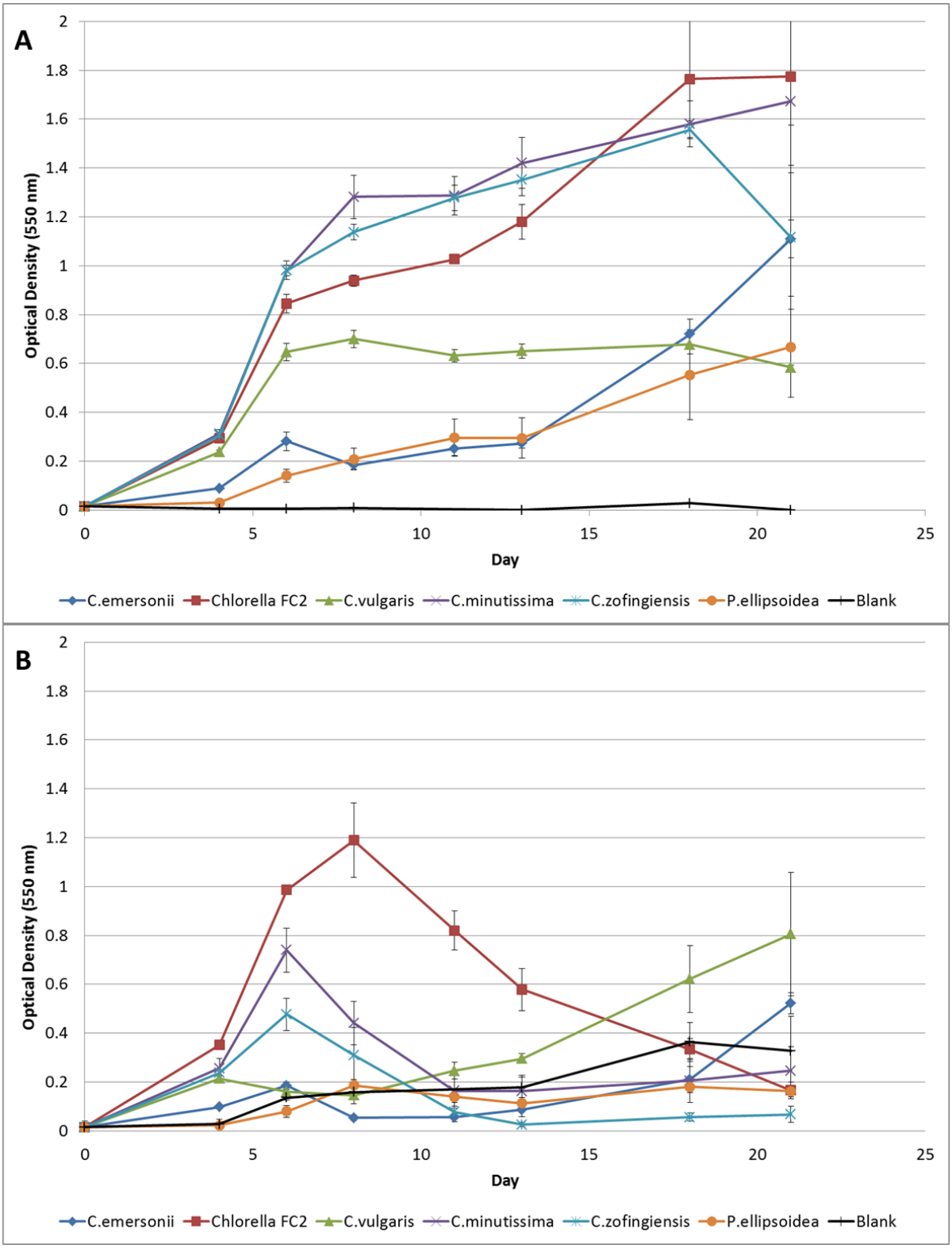

Figure 5.3. Optical density at 550 nm of six species of microalgae and blank (no addition of algae) in: A, sterile (autoclaved) and B, non-sterile wastewater final effluent, n=3, error bars represent ± 1 SE.

Flow cytometry cell counts have shown a variable effect of WW sterility on the growth and biomass accumulation of the algal species screened. *C. zofingiensis* and *C. vulgaris* were almost eliminated from the cultures by day 13 and day 8 respectively, while *C. minutissima* showed a similar pattern to *C. zofingiensis* albeit with cells remaining present throughout the experiment at concentrations above $0.5*10^6$ cells ml^{-1}. *Chlorella* FC2 IITG initially showed rapid growth in the non-sterile treatment but after day six the cell concentration in the non-autoclaved WW started dropping and continued to do so until the end of the experiment. Contrary to the above four species, *C. emersonii* and *P. ellipsoidea* showed very similar growth curves in the autoclaved and non-autoclaved treatments.

One of the main differences that was observed between the two WW treatments was the presence of biofilms in all non-autoclaved WW cultures despite being shaken at 120 rpm. To minimise the extent of biofilm formation all flasks were, in addition, vigorously swirled daily, however they persisted regardless in most bottles. This means that our measurements may have underestimated the productivity of non-autoclaved WW cultures as a significant proportion of productivity may have been locked in biofilms.

From our experiments certain conclusions can be drawn: all six species used here were able to grow in autoclaved WW. In non-autoclaved WW adding a species of algae did not, in most cases, improve productivity when compared to the blank treatment where the native community was allowed to bloom. The species that showed the highest growth rates (namely *Chlorella* FC2 IITG, *C. minutissima* and *C. zofingiensis*) were eliminated from the non-autoclaved WW cultures or were present at low concentrations, while the *C. emersonii* and *P. ellipsoidea* which had the lowest growth rates, did not show a significant difference in cell numbers between autoclaved and non-autoclaved WW. Finally, complex and variable interactions between species are likely to take place as suggested by the cyanobacterial dominance in two out of three non-autoclaved *C. vulgaris* cultures (see Appendix 8.2).

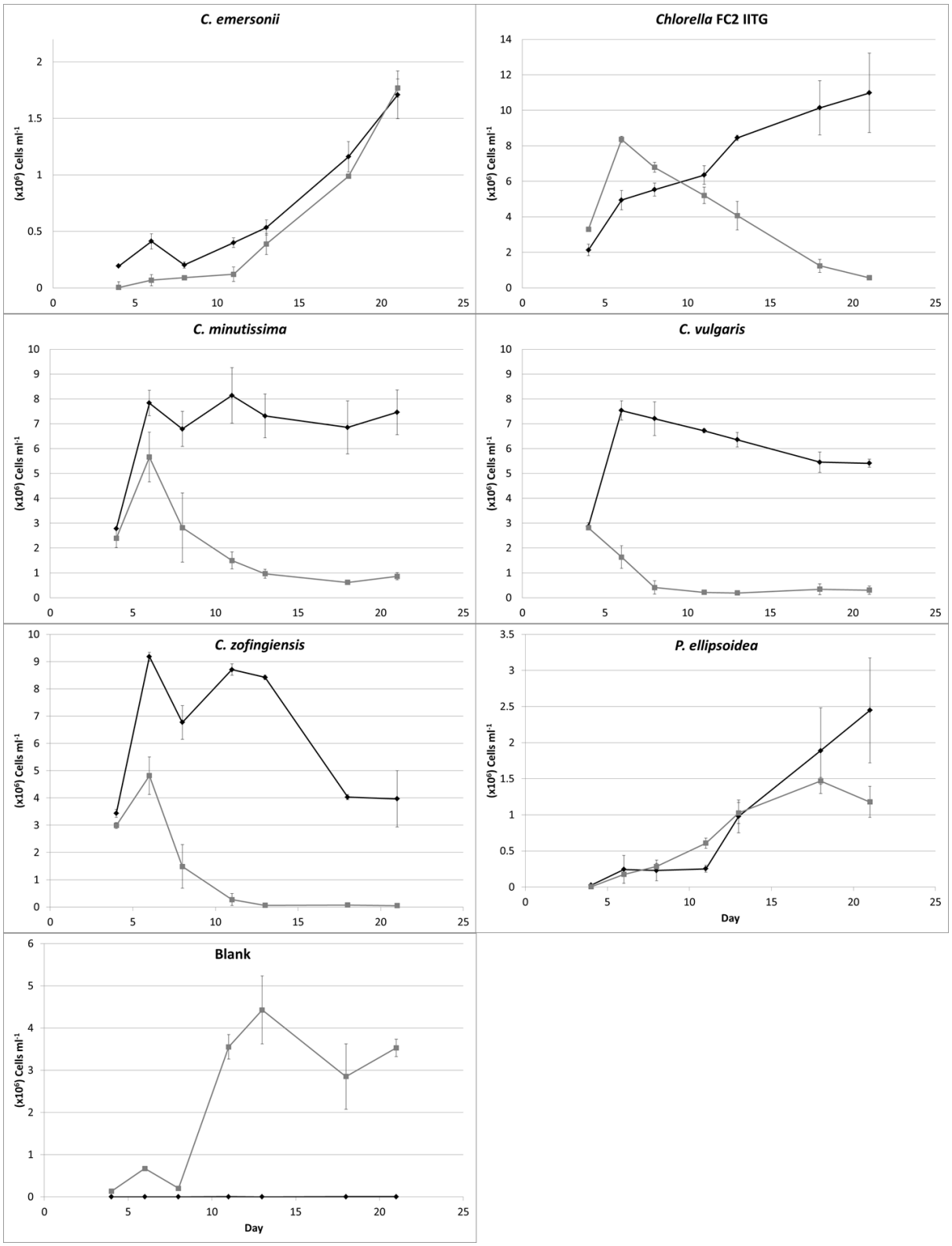

Figure 5.4. Cell counts by flow cytometry of viable algal cells in autoclaved (black line) and non-autoclaved (grey line) wastewater final effluent for six species of microalgae and blank with no addition of algae. Error bars represent ± 1 SE (n=3).

5.3.2 *Mechanical strength of algal cell walls as revealed by ultrasonication.*

We grew six species of algae in triplicate as described above (section 2.2.1.6) to assess mechanical cell wall strength by ultrasonication and cell wall thickness by TEM and image analysis in two different growth phases, namely late exponential and stationary. For late exponential growth phase, algae were harvested at days 7-9 and for stationary phase they were harvested on days 18-20 (fig. 5.5.).

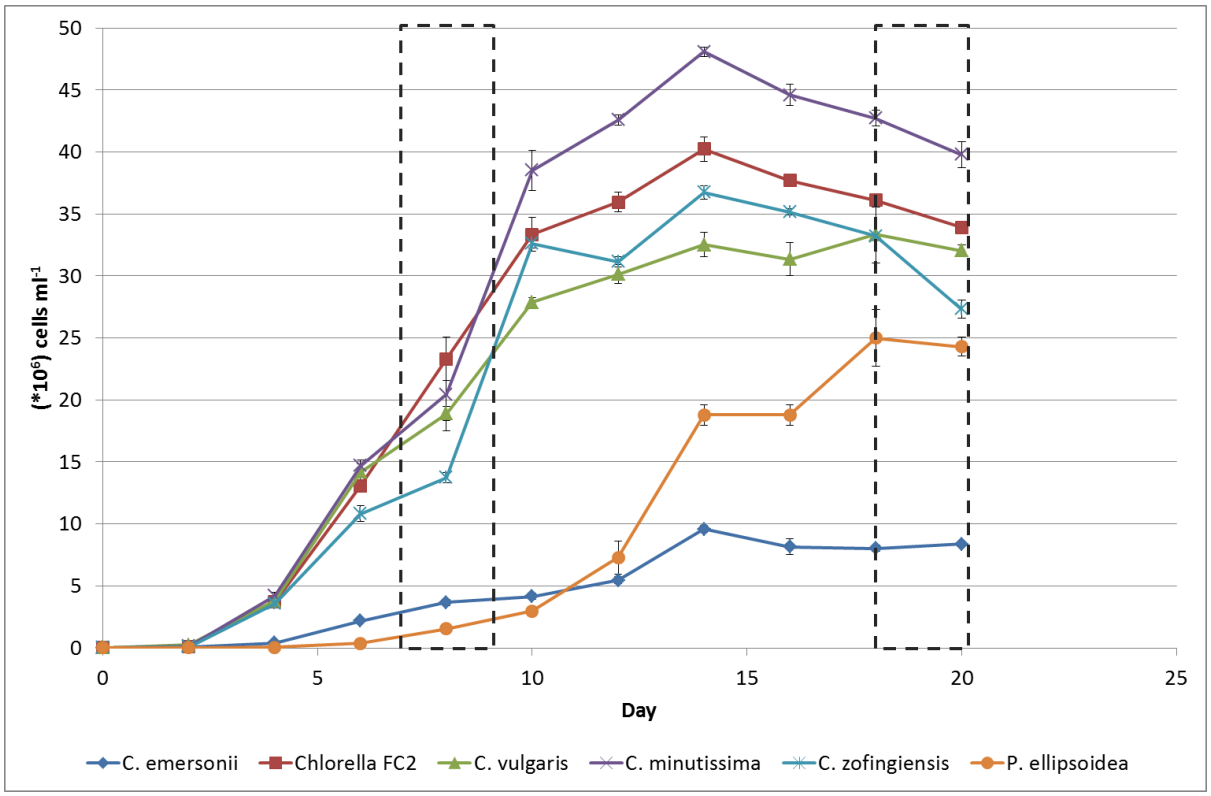

Figure 5.5. Growth curves of six species of microalgae in BBM/4 medium for cell disruption by ultrasonication. n=3, error bars represent ± 1 SE. Dashed line rectangles show the time of harvesting for ultrasonication; specifically cells were harvested on days 7-9 and days 18-20 for ultrasonication at late exponential phase and stationary phase respectively.

Cell disruption by ultrasonication in 1 min bursts for 15 minutes total (fig. 5.6.) showed that *C. emersonii* was the most resistant alga to ultrasonication while the four "true *Chlorellas*", *C.* FC2 IITG, *C. minutissima, C. vulgaris* and *C. zofingiensis* had similar disruption patterns. *P. ellispoidea* was the most easily disrupted alga. The above is true for both growth stages.

However for each species, the stationary phase algae showed an increase in mechanical cell wall strength as revealed by resistance to disruption by ultrasonication.

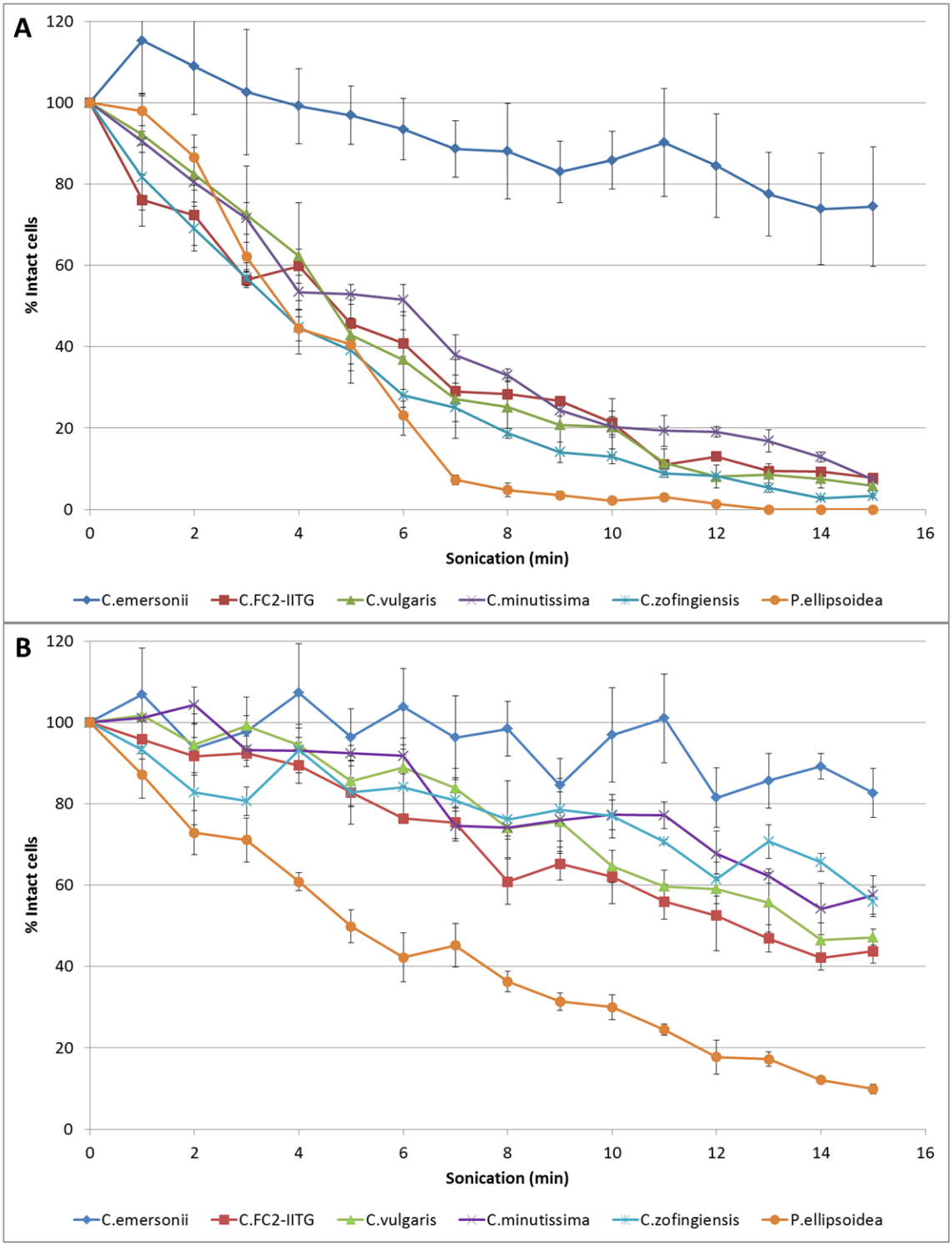

Figure 5.6. Cell disruption by ultrasonication of six species of microalgae at: A, late exponential growth stage (days 7-9) and B, stationary stage (days 18-20). Intact cells were assessed by microscopy counts in 0.4% trypan blue compared to the control (before ultrasonication). Ultrasonication was performed in one minute bursts with four minutes rest on ice. Error bars indicate ± 1 SE (n=3).

Figure 5.7 shows the percentage of undisrupted cells after 15 minutes of ultrasonication for each species at both growth stages. With the exception of *C. emersonii* which was highly resistant to cell disruption in both growth phases, all other species showed a highly significant ($p<0.001$) increase in the percentage of undisrupted cells after 15 minutes of ultrasonication. In particular, with the exception of *C. emersonii*, late exponential species showed levels of intact cells in the range of 0 % (*P. ellipsoidea*) – 8.8 % (*C.* FC2-IITG) after 15 minutes of ultrasonication while in stationary phase the percentage of intact cells ranged from 11.2 % (*P. ellipsoidea*) to 62.1 % (*C. minutissima*) (fig. 5.7). Furthermore, the presence of algaenan did not seem to have a significant effect on the mechanical strength of the cell wall as both the strongest and the weakest walled species had a confirmed presence of algaenan. Our data, therefore, suggests that the exact point of harvesting is critical to the downstream costs of cell disruption and that there may be a double trade-off in nutrient starvation for lipid accumulation where not only the whole system becomes less productive but also costs for cell disruption are significantly increased. It is also important to note that 15 minutes of ultrasonication resulted in complete cell disruption in only one case (*P. ellipsoidea* – late exponential phase). This shows that algal cell walls are generally very resistant to disruption by ultrasonication.

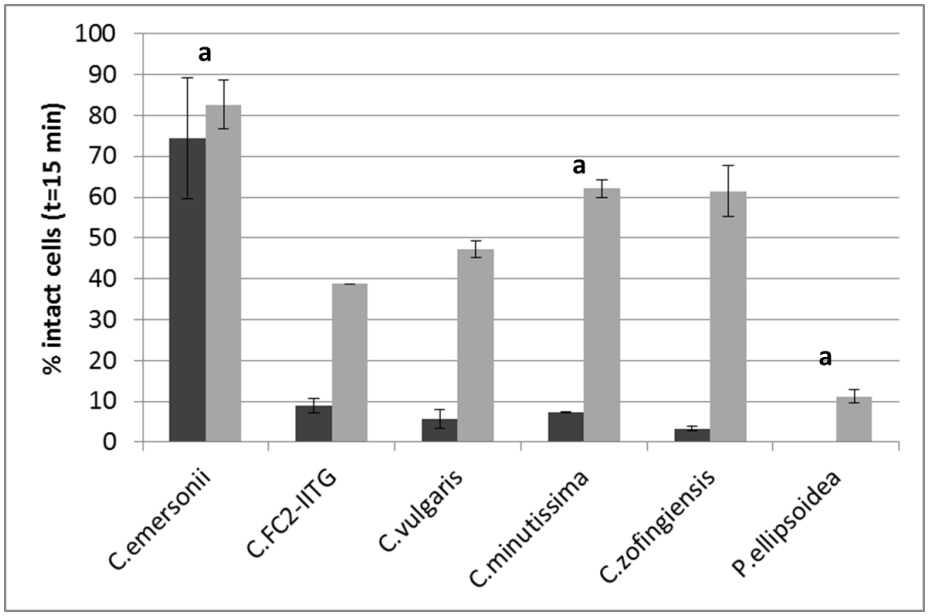

Figure 5.7. Percentage of intact cells of six species of microalgae after 15 minutes of ultrasonication at late exponential (dark grey bars) and stationary (light grey bars) growth stages. Values represent averages of three independent cultures (n=3) and error bars represent ± 1 SE. "a" denotes the presence of algaenan in a species.

It has been previously shown that a some algal species undergo changes in cell wall ultrastructure when entering the stationary stage (Hagen *et al.*, 2002; Van Donk *et al.*, 1997). We therefore decided to investigate further whether the increased resistance to disruption in stationary phase could be correlated to a change in cell wall ultrastructure and specifically cell wall thickness as revealed by TEM images (Appendix 8.1.). Image analysis showed that cell wall thickness was always higher in stationary phase than in late exponential although this difference was not always significant (fig. 5.8.). However, growth stage had a significant effect on cell wall thickness overall (ANOVA, $p<0.002$).

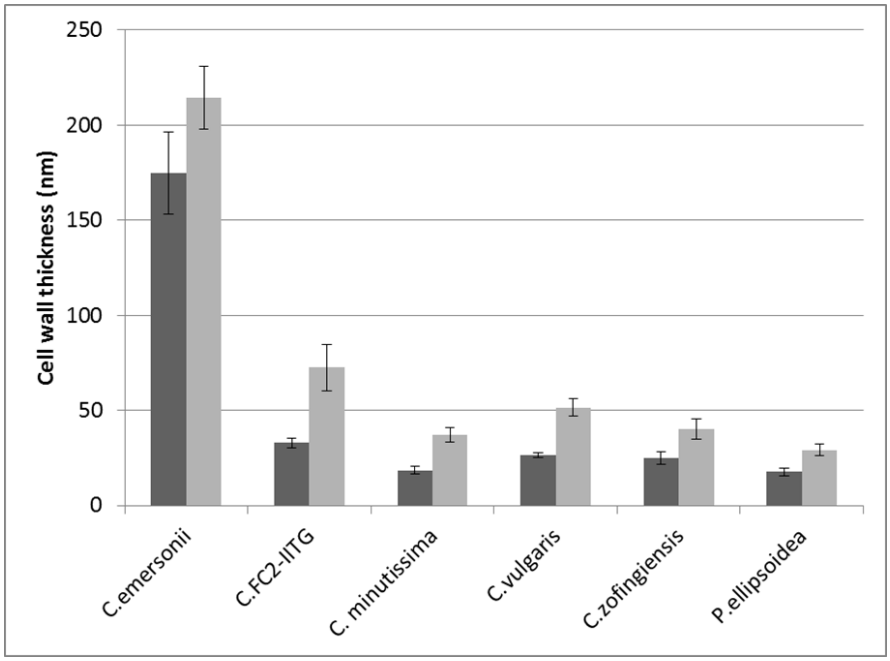

Figure 5.8. Cell wall thickness of six species of microalgae in late exponential (dark grey) and stationary (light grey) growth stages. Values represent average thickness of five cells, measured by image analysis of TEM pictures, error bars represent ± 1 SE.

To reveal whether changes in cell wall thickness could explain the increase in cell wall strength, we calculated the ultrasonication LD_{50} (the time of ultrasonication required to disupt 50% of cells) by fitting an exponential curve to each of the cell disruption curves in figure 6 and calculating the LD_{50} from the resulting equation. LD_{50} was then plotted against cell wall thickness and carried out a linear regression analysis (fig. 5.9.). This showed that ultrasonication LD_{50} could be explained reasonably well by changes in the cell wall thickness independently of species or growth stage (r^2=0.85, $p<0.001$). We therefore concluded that, within the Chlorophyte algae assessed in this experiment, cell wall thickness and not the production of algaenan or the growth stage *per se* is the most important factor to predict cell disruption costs.

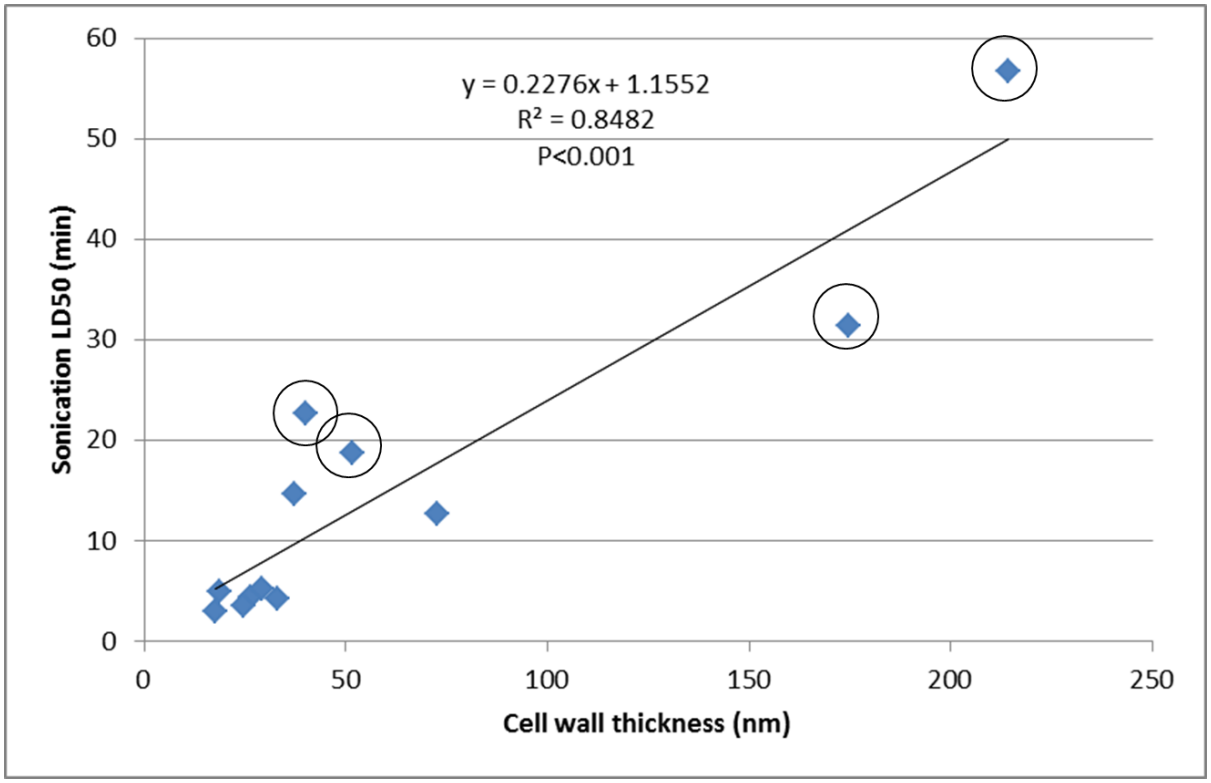

Figure 5.9. Regression of ultrasonication LD50 against cell wall thickness for six species of microalgae in late exponential and stationary phase. LD_{50} values were calculated from the exponential regression equations for each species. Circled values were not reached experimentally (LD_{50} > 15 min).

It is also worthwhile to note that all the species studied had a spherical shape with the exception of *P.ellispoidea* which has an elliptical shape. The fact that this species was the most sensitive to cell disruption (fig. 5.6.A,B and fig. 5.7.) while having only slightly thinner cell walls (fig. 5.8.) may suggest that shape is also an important factor in predicting the cell disruption costs but our experiment was not designed to investigate this parameter.

5.3.3 Sequential lipid extraction kinetics and lipid quantification.

To assess how cellular characteristics such as algaenan production, cell wall thickness, cell size and lipid content might affect lipid extraction kinetics we extracted lipids from undisrupted, stationary stage (day 20), lyophilised biomass from six-species of green microalgae and full fat soybean flour. The later was used as a useful comparison to a terrestrial biodiesel source and because it has been assumed in various life-cycle analyses that lipid

extraction from microalgae has the same costs as soybean lipid extraction (Lardon *et al.*, 2009; Xu *et al.*, 2011; Yang *et al.*, 2010).

Extractions were performed using chloroform/methanol (2:1 vol/vol) at room temperature. The solvent was completely replaced every 45 minutes up to 3 hours and at 24 h and 48 h and lipid content was quantified at these time points. Lipids were considered to be fully extracted when the weight increase from the previous time point was less than 2% of dry weight. Lipid from soybean flour was fully extracted after 135 min, while *C. vulgaris, C.* FC2 IITG and *C. zofingiensis* required 24 h of extraction and *C. emersonii*, *C. minutissima* and *P. ellipsoidea* required 48 h of extraction before an increase of less than 2 % was observed. Final lipid contents were 24.7 % for full fat soybean flour, 35.1 % for *C. emersonii*, 41.8 % for *P. ellipsoidea*, 50.4 % for *Chlorella* FC2 IITG, 58.1 % for *C. vulgaris*, 59.8 % for *C. zofingiensis* and 63.6% for *C. minutissima* (fig. 5.10.A). Because of the highly variable lipid content between species, we also plotted lipid extraction kinetics as a function of the percentage of total lipid content (fig. 5.10.B) and performed the comparisons on this data set. This "normalisation" of the data is valid because of the large s/b ratio (75 ml solvent for 0.5 g biomass) and the sequential nature of the extraction, which means kinetics are minimally affected by the difference in lipid content of different species and the rate limiting step becomes solvent penetration through the cell wall rather than the lipid concentration gradient.

Soybean flour was the most easily extractable biomass of this study as lipid was 96.8% extracted within the first 45 minutes. At the same time point our algal species showed highly variable extraction yields ranging from 35.6% (*C. minutissima*) to 76.1% (*C. vulgaris*) of total lipid (fig. 5.11.). We attempted to correlate extractability in the first 45 minutes to cellular characteristics such as cell wall thickness, cell volume and lipid content but found no correlation ($r^2<0.2$ in all cases, data not shown). The only characteristic that seemed to predict extractability in the first 45 minutes was the presence of algaenan. Figure 5.11 shows that the three species with confirmed algaenan

production had the lowest extractabilities among our screen irrespectively of lipid content. Therefore our data suggests that algaenan cell walls form a considerable barrier to solvent penetration when compared to the polysaccharide walls found in the other species leading to lower extractability.

By 180 minutes of sequential extraction (four solvent changes) our six algal species showed extraction yield between 90.4 % (*C.* FC2 IITG) and 99.4 % (*C. vulgaris*) with the notable exception of *C. emersonii* which was only 70.8 % extracted by that time point. Furthermore, a visual examination of figure 5.10.B showed that the relationship between algaenan production and extractability had changed, that is the three algaenan producers no longer had the lowest extractabilities.

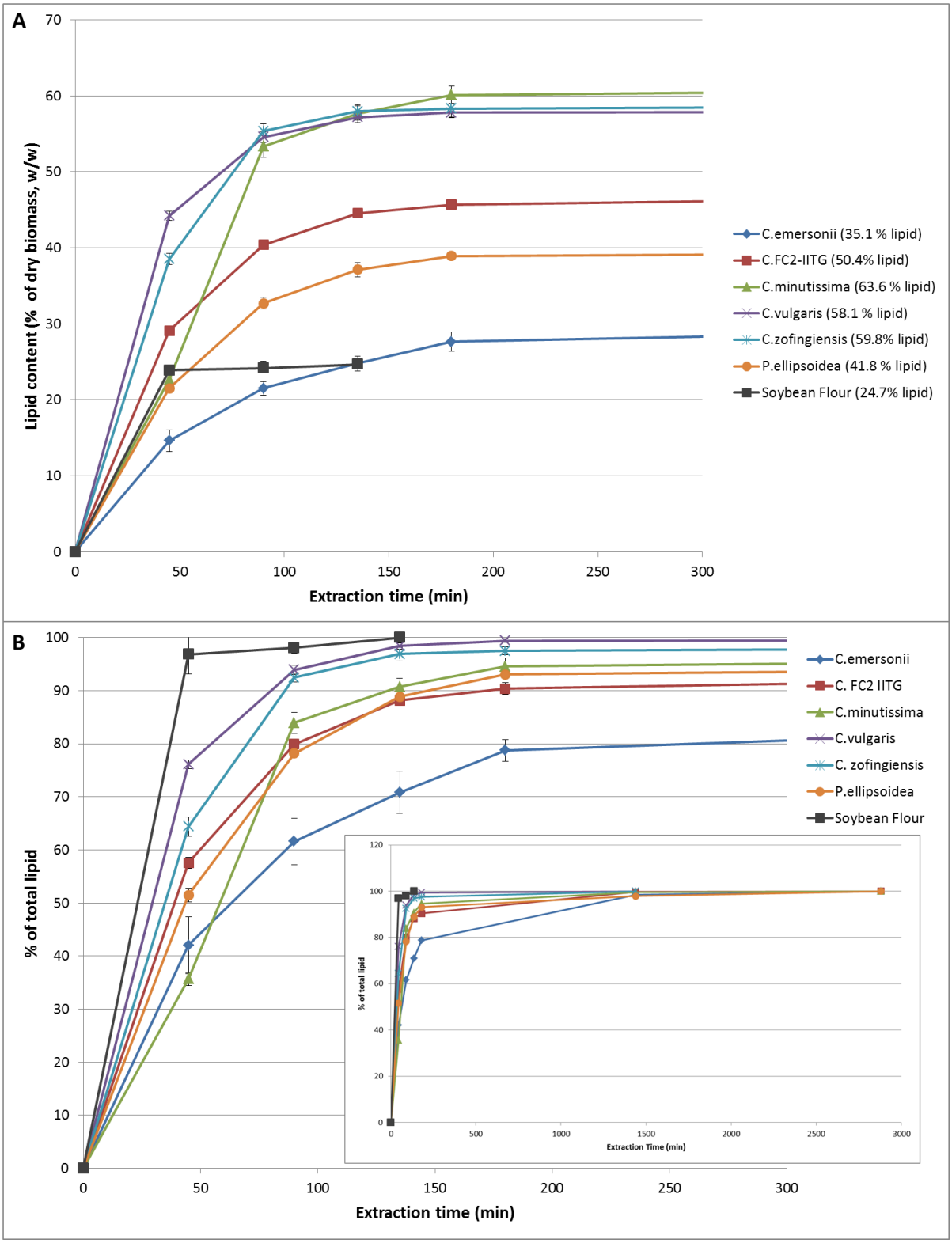

Figure 5.10. Lipid content as percentage of dry biomass, (A) and lipid extraction kinetics as percentage of total lipid, (B) of six species of microalgae and full fat soybean flour extracted by sequential chloroform:methanol (2:1 vol/vol) extraction at room temperature. Values represent averages of three extractions from a single 8 L vPBR culture, error bars represent ± 1 SE. Final lipid contents (24-48 h extractions) are presented in the legend of (A). The inset in graph (B) shows the full 48 h extraction.

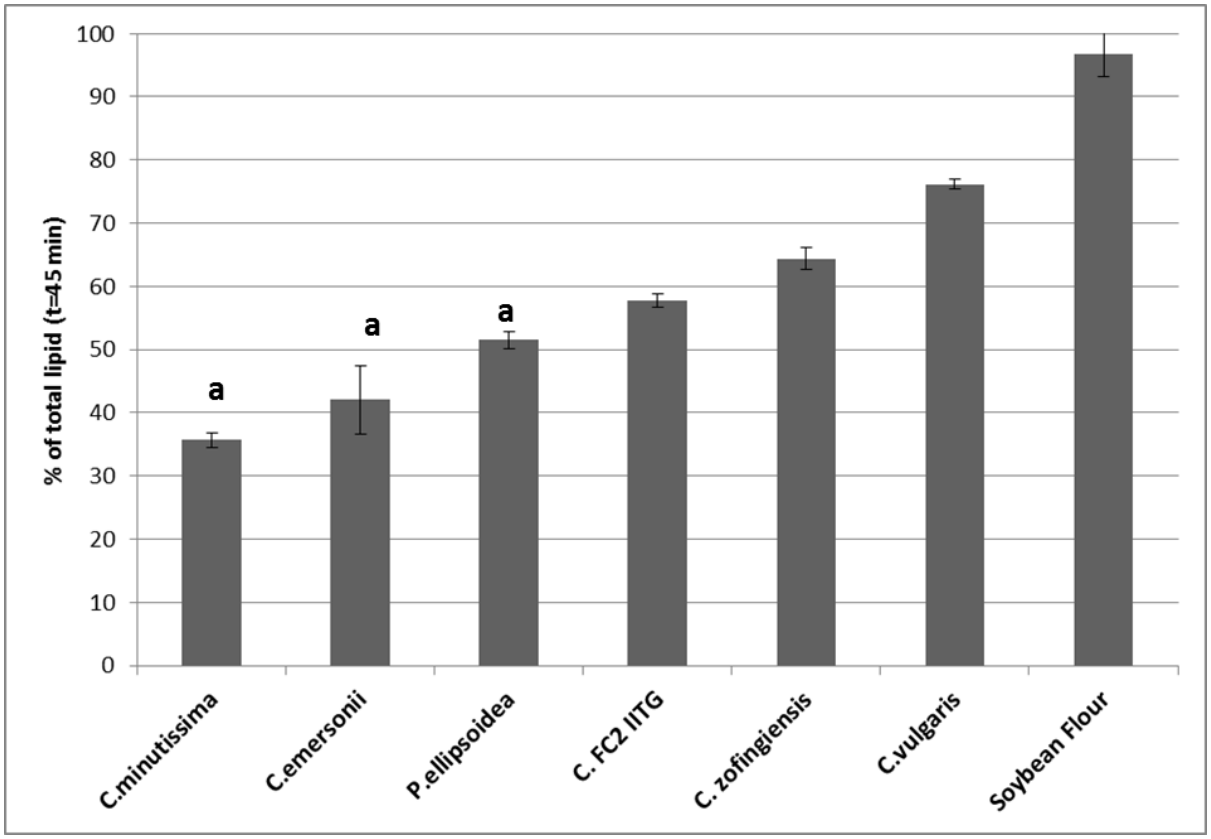

Fig. 5.11. Percentage of total lipid extracted from six species of microalgae and full fat soybean flour in the first 45 minutes of extraction. Lower case "a" above values represents the proven production of algaenan. Values represent averages of three extractions from a single 8 L vPBR culture of each species and a single batch of Soybean flour. Error bars represent ± 1 SE.

We carried out the same exploratory linear regression analyses (Minitab, Fitted Line Plot) for the 180 minute time point and found that extractability now showed a good correlation with cellular characteristics (fig. 5.12.). When only one predictor was used, extractability at 180 minutes best correlated with cell wall thickness (r^2=0.83, p<0.013, S=3.42). When two predictors were used, cell wall thickness divided by cell size gave the best correlation (r^2=0.85, p<0.009, S=3.14) and finally, when all three predictors were used, the best correlation was against "cell wall thickness/ (cell size * lipid content)" (r^2=0.88, p<0.006, S=2.86).

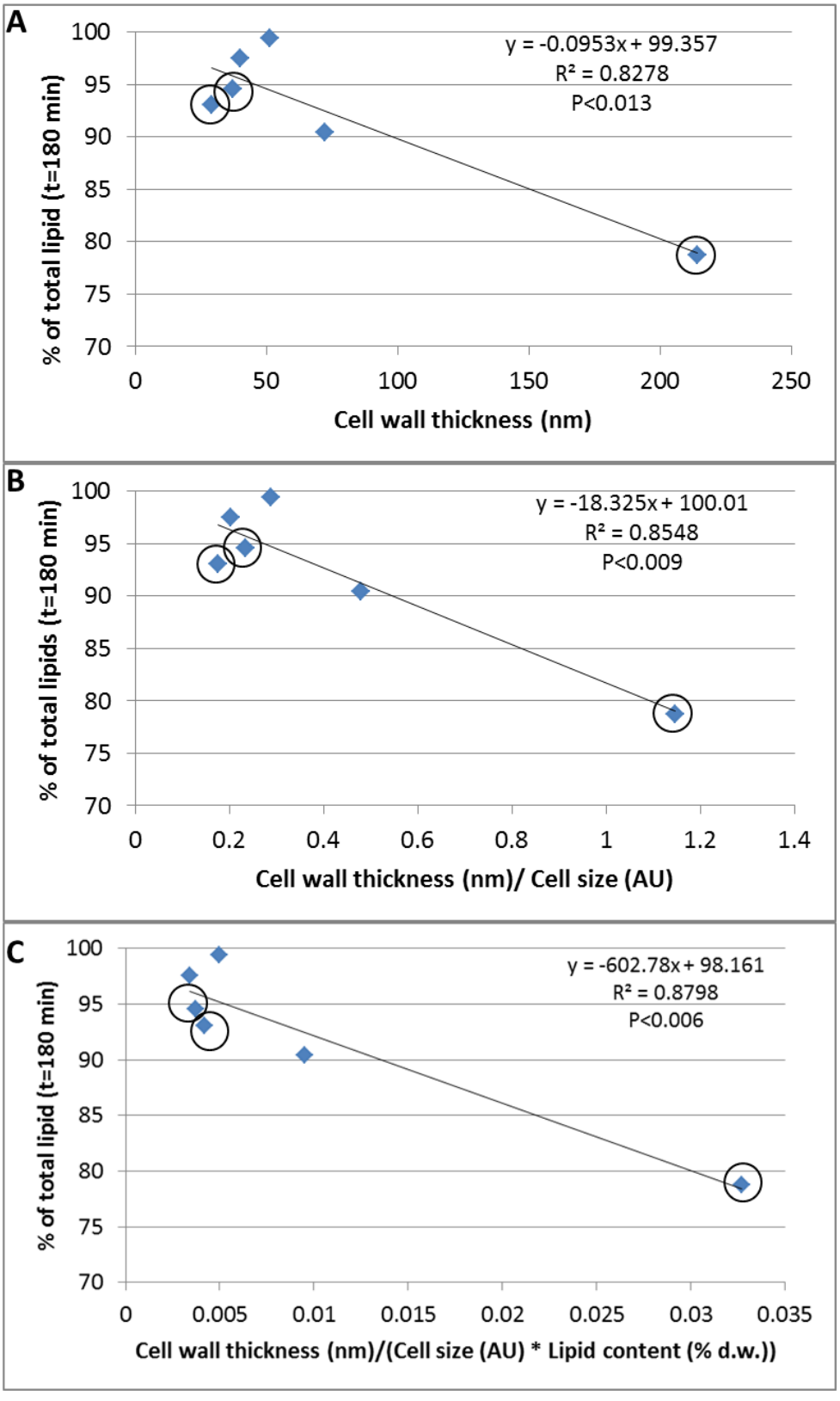

Figure 5.12. Regression of percentage of total lipids extracted from six species of undisrupted microalgae in stationary phase (day 21) against; A, cell wall thickness B, cell wall thickness divided by the relative cell volume as measured by flow cytometry (FCS Area) and C, cell wall thickness divided by cell volume times lipid content. Data points in circles represent algaenan production.

This suggests that although algaenan is relatively impermeable to organic solvents in the first 45 minutes of extraction, chloroform/methanol eventually permeabilises this cell wall material and so by 180 minutes of extraction the production of algaenan is not a predictor of extractability. Furthermore our data suggests that cell wall thickness is a good predictor of extractability, at least within the Chlorophyte algae, and that a proxy for cell size and the total lipid content were also useful in increasing the strength of this prediction as shown by the increase in r^2 and decrease in p-value and standard error (S-value) of the regression. It is important to note that while in a multiple regression model adding more factors almost always increases the r^2 value as the software adds its own constants in front of each predictor to make the data fit as well as possible, in our case the increase in r^2 is not an artefact as we calculated only simple ratios between the different predictors. Therefore we propose that when operating variables are kept constant and species are varied, the lipid extraction mass transfer constant k is a function of cell wall composition, cell wall thickness, cell size (within a given cellular shape or surface area to volume relationship) and total lipid content. This can be expressed as:

$$k = f(W_m, W_t, S, L_t)$$

Where k is the lipid mass transfer constant, W_m is a coefficient to account for the permeability of the cell wall material, W_t the total cell wall thickness, S is the cell size (within a given cell shape) and L_t is the total lipid content.

5.3.4 Elemental and biochemical analysis and high heating values (HHV).

Biomass from the same 8L vPBR cultures that were used for lipid extraction was subjected to a full elemental and biochemical analysis (summarised in table 5.1). Moisture content of lyophilised biomass ranged between 1.5 % and 1.7% with the exception of *C. emersonii* which had 3.1 % moisture. This suggests that *C. emersonii* was more resistant to freeze-drying

than the other species in our screen and this may be explained by the fact that this species had by far the thickest cell wall (fig. 5.8.) as well as being an algaenan producer. *C. emersonii* also had the lowest C content (54.6 % of dry ash-free weight (daf)), lowest lipid content (27.6 %) and HHV values (21.4 MJ/kg) of all species. The other species showed a carbon content ranging between 58 % daf (*P. ellipsoidea*) and 65.7 % daf (*C. vulgaris*) and HHV between 28.5 MJ/kg (*P. ellipsoidea*) and 34.6 MJ/kg (*C. vulgaris*). All strains had a low protein content ranging between 8.5 % (*C. emersonii*) and 10.5 % (*C. zofingiensis*). The low protein content seen here is due to culturing these species well into stationary phase (21 days) and in a low-N medium (BBM/4) which means that the six species had undergone nitrogen starvation for several days (approximately 6 ± 1 days of nitrogen starvation as judging from growth curves, data not shown). Finally, it is worthwhile noting that the lipid content in this extraction is lower than the lipid content as revealed by sequential lipid extraction. This is likely an artefact of a less exhaustive method employed for lipid extraction in the biochemical analysis (Folch method), thus the lipid contents described earlier are closer to the real lipid content of our species.

Table 5.2. Analysis of lyophilised biomass of six species of green microalgae; *Chlorella emersonii* (CE), *Chlorella* FC2 IITG (CF), *C. minutissima* (CM), *C. vulgaris* (CV), *C. zofingiensis* (CZ) and *Pseudochoricystis ellipsoidea* (PE).

			Dry ash free %						as received %		
Species	Ash %	Moisture %	C	H	N	S	O	HHV (MJ/kg)	Carbo-hydrate	Lipid	Protein
CE	2.8	3.1	54.6	6.6	1.8	0.4	36.6	21.4	35.7	27.6	8.5
CF	4.3	1.7	62.0	10.7	2.2	0.3	24.9	31.8	23	35.1	9.8
CM	3.3	1.5	62.5	9.6	2.2	0.5	25.2	30.5	13.2	54.0	9.8
CV	8.2	1.5	65.7	11.2	2.2	0.3	20.6	34.6	11.5	52.4	9.4
CZ	4.8	1.6	63.0	10.9	2.4	0.4	23.3	32.7	10.8	53.1	10.5
PE	7.4	1.7	58.0	9.9	2.1	0.1	29.8	28.5	31	34.6	9.3

5.3.5 Hydrothermal liquefaction.

Hydrothermal liquefaction (HTL) is a process that has recently gained hugely in popularity among algal biofuel companies (Liu *et al.*, 2013) and researchers (Jazrawi *et al.*, 2013; Torri *et al.*, 2012; Valdez *et al.*, 2012) mainly because of its simplicity, scalability and the fact that it avoids the problems of dewatering, cell disruption, lipid extraction and transesterification in a single step by using sub-critical water to convert the whole wet biomass into a bio-crude oil (Barreiro *et al.*, 2013a). We investigated the suitability of our six-species screen to HTL as an alternative process to lipid extraction. HTL was performed on lyophilised biomass from the same batch that was used for lipid extraction, at 350 °C for 15 minutes.

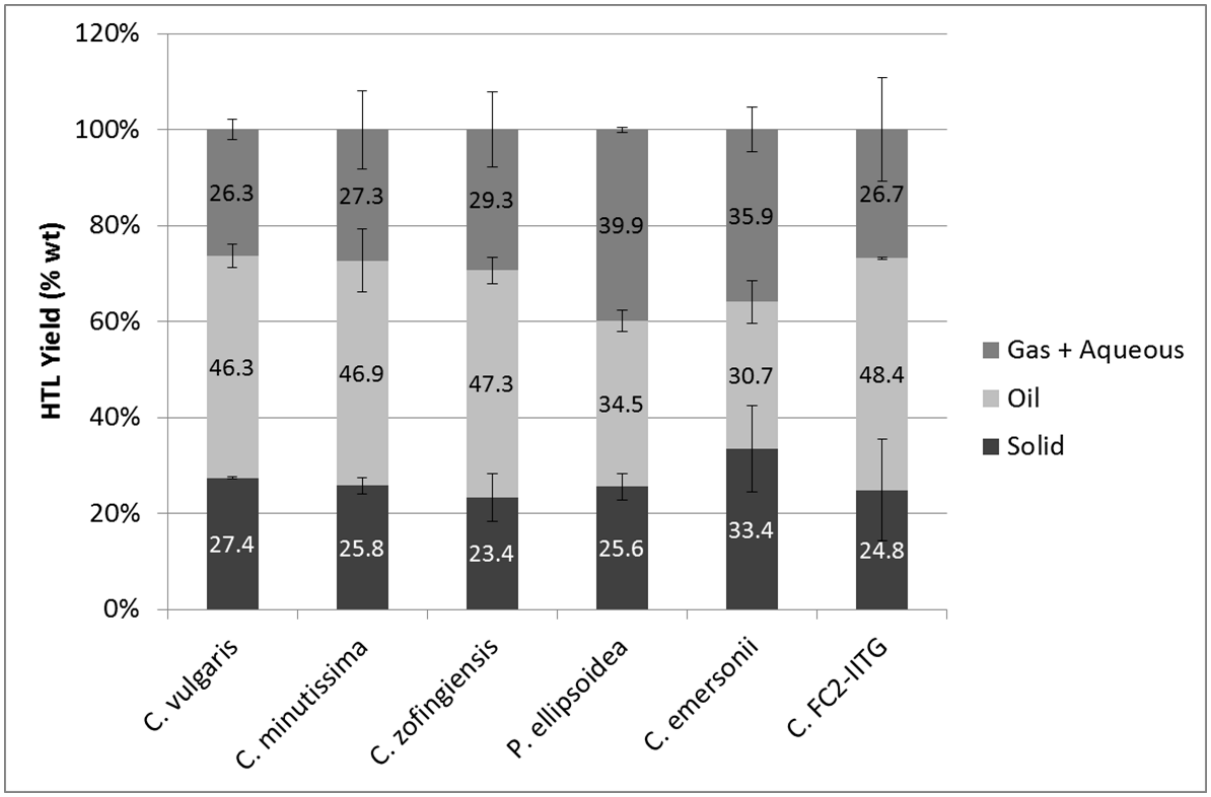

Figure 5.13. Product balance of HTL to different phases for six species of green microalgae. Error bars represent ± 1 SD. The gaseous phase was not measured and therefore was combined with the aqueous phase so that the sum of all phases was 100 %. All other products were measured.

The product balance (fig. 5.13.) shows that all "true" *Chlorella* species grouped very tightly together giving an oil yield between 46.3 % dw (*C. vulgaris*) and 48.4 % dw (*C.* FC2 IITG) and solid yields between 23.4 % (*C. zofingiensis*) and 27.4 % (*C. vulgaris*). *P. ellipsoidea* and *C. emersonii* gave biocrude yields of 34.5 % and 30.7 % respectively as well as solid yields of 25.6 % and 33.4 % respectively. The presence or absence of algaenan did not have any effect on the oil or solid yields of the six species but the initial oil yields show some correlation with the HTL oil yields (r^2 =0.797, data not shown). In all cases a fairly high percentage of solid residue (fig. 13) was obtained when compared to the ash content of the biomass (table 1) and the available literature (Barreiro *et al.*, 2013b; Biller *et al.*, 2012; Jazrawi *et al.*, 2013). This is probably due to incomplete liquefaction of the algal biomass and suggests that HTL should be further optimised for these species, that is higher temperature and/or longer residence times were needed for the complete degradation of the biomass. Oil yields were surprisingly low, considering the very high lipid content of the biomass as revealed by sequential lipid extraction (fig. 5.10.A). This could be due to suboptimal HTL performance leaving part of the lipid associated with the solid phase and/or due to gasification of the more volatile portion of lipids.

The characteristics and elemental analysis of the bio-crude obtained are summarised in table 5.2. The most striking result in our HTL experiment was the very low N content of the biocrude oil. All species ranged between 1.1 % (*C. minutissima*) and 1.5 % (*C. emersonii*) with the exception of *P. ellipsoidea* which had a 1.8 % N content. The N content of HTL biocrude is generally between 3.2 % and 7.1 % (reviewed by Barreiro *et al.*, 2013) and only one published study (Dote *et al.*, 1994) on *Botryococcus braunii* has showed a lower N content (0.7 %). This is important because high nitrogen content in the biocrude oil is one of the biggest problems with current HTL methods (Barreiro *et al.*, 2013a) meaning that the biocrude needs pretreatment/upgrading before it can be used and, more importantly, nitrogen lost into the oil phase cannot be recycled as a nutrient and thus increases the demand for fertilisers or other nutrient import. The loss of

nitrogen into the oil can be one of the most energetically costly factors affecting algal-HTL biofuel (Liu *et al.*, 2013). It is also notable that all six of our species had sulphur content below detection, with the exception of *C. emersonii* (0.1 % S). This also reduces the need for pre-treatment (desulphurisation) below that of fossil crude oils that have a significant presence of sulphur (Liu *et al.*, 2013). Our data suggests that by letting microalgae grow into stationary stage and undergo nutrient starvation for a few days prior to harvesting, it is possible to lower the nitrogen content of the HTL biocrude to more acceptable levels and almost eliminate sulphur from the biocrude.

Table 5.3. Yield (% of dry, ash-free biomass), elemental analysis, HHV and % energy recovered of bio-crude oil obtained by hydrothermal liquefaction (350 °C, 15 mins) of six species of green microalgae; *Chlorella emersonii* (CE), *Chlorella* FC2 IITG (CF), *C. minutissima* (CM), *C. vulgaris* (CV), *C. zofingiensis* (CZ) and *Pseudochoricystis ellipsoidea* (PE). Daf = dry, ash-free weight.

		Oil ultimate (%)						
Species	Oil yield (daf %)	C	H	N	S	O	HHV (MJ/kg)	% Energy Recovery
CV	51.2	76.1	11.2	1.2	0.1	11.5	39.8	58.9
CM	49.3	72.7	9.6	1.1	0.0	16.6	35.3	57.1
CZ	50.5	68.9	10.0	1.2	0.0	19.9	34.0	52.6
PE	38.0	75.4	10.6	1.8	0.0	12.2	38.5	51.4
CE	32.6	71.7	9.8	1.5	0.0	17.1	35.2	53.7
CF	51.5	73.2	10.8	1.4	0.0	14.6	37.6	60.8

5.4 Discussion.

5.4.1 Anaerobic digestate and municipal wastewater as nutrients for microalgal biofuel production.

We used autoclaved and non-autoclaved municipal wastewater and diluted anaerobic digestate to assess the growth and suitability of six species of green oleaginous algae to these "free" nutrient sources. In an ideal, industrial scale algae plant, the majority of nutrients required for growth would be directly recycled on site by breaking down the remnants of the algal

biomass after all valuable products have been extracted (Liu *et al.*, 2013; Sialve *et al.*, 2009). This is possible either by anaerobically digesting the spent algal biomass and using the diluted anaerobic digestate as a nutrient source (Erkelens *et al.*, 2014) or by using the diluted aqueous phase of hydrothermal liquefaction (Biller *et al.*, 2012). However in each case it is practically impossible to recycle 100 % of the nutrients so some nutrients have to be imported to supplement for inherent systematic losses. Fertilisers, and especially ammonia, are very energy intensive to produce as well as having very high CO_2 emissions (Clarens *et al.*, 2010) to the point that the requirement for ammonia fertiliser becomes one of the most significant energy burdens in life-cycle studies of microalgal biofuel (Liu *et al.*, 2013). The desire to avoid the use of fertiliser has led to the investigation of anaerobic digestate and various wastewaters as "free" nutrient sources.

Our study shows that autoclaved ADM and WW are good growth media for freshwater Chlorophyta. However maintaining axenic conditions in an industrial scale production facility for such a low-price product as biofuel is pragmatically unachievable; therefore we also investigated the non-autoclaved version of the above media to assess algal growth when the native community is alive. AD proved to be best medium in the non-autoclaved condition because growth rates were only slightly reduced or similar to the autoclaved condition. This is likely because of the characteristics of anaerobic digestion as a process; the anaerobic nature and the specificity of the resident bacterial community as well as the higher than ambient temperature of this process means that any microorganisms present in the AD growth media will have sub-optimal growth rates and are unlikely to be strong competitors for nutrients or organic carbon present. Algal predators or parasites can still be introduced from the environment in open systems but at least their absence is virtually guaranteed in the nutrient feed, even without the need for sterilisation.

In contrast non-autoclaved WW was a very challenging medium with variable results for our six species screen and even within replicates of the

same species; the extensive formation of biofilms hindered suspended growth rates in all but one species (*C. emersonii*) and none of the treatments performed significantly better than the "blank" condition (negative control; no algae added) where native WW algae were allowed to bloom. This suggests that native species sampled directly from the WW may perform better than species selected for high growth rate and lipid content however this would have a negative effect on the productivity of an algal biofuel plant. Furthermore, the very complex nature of the WW community, which included a number of species of coccoid, colonial and filamentous green algae as well as diatoms, cyanobacteria and fungi among others, makes the behaviour of the system unpredictable and prone to culture crashes or at least considerable fluctuations in productivity. Algae may still be good bioremediators of WW in a dual purpose WW treatment/biofuel systems (Craggs *et al.*, 2012) but if biofuel/bioenergy production is the main focus (as in the present study), our results show that WW should be avoided as a complimentary nutrient source. Furthermore the necessary co-location of a biofuel plant next to WW treatment works would possibly impose unwelcome restrictions in land availability and land price making this an unattractive scenario for algal biofuel production.

It is however possible to imagine a fully integrated algal production facility where the extracted biomass is turned to methane by anaerobic digestion and the resulting anaerobic digestate is recycled as a nutrient source for the growing algae. In this case a limited import of external agricultural, food or other solid, digestible waste would be the only activity required to supplement for nutrients lost in the process of recycling. This process would provide an environmental service as well as a boost to the methane production of the facility and finally, different materials could be investigated in co-digestion studies to optimise the digestate for algal growth. However, so far only one study has recycled anaerobically digested algae as a growth medium for the same species (Erkelens *et al.*, 2014) and this was performed on whole, unprocessed algal biomass.

As result of the above, we believe it essential for the algal research community to investigate and optimise the integration of microalgae production and anaerobic digestion more actively in the future. In particular, studies are needed where algal products are extracted using solvents with and without prior cell disruption and the extracted biomass is anaerobically digested or co-digested with various solid waste products with the resulting anaerobic digestate being recycled for algal growth. Such studies and the optimisation of the above processes would greatly help in realising the potential of a, theoretically, sound, integrated path to microalgal biofuels that would solve the nutrient supplementation problem.

5.4.2 The importance of the algal cell wall in cell disruption and implications for downstream processing.

We assessed cell disruption by ultrasonication in six species of Chlorophyte microalgae in late exponential and stationary growth stage to assess how the production of algaenan and cell wall thickness affect cell disruption kinetics. We found that the growth stage had a significant and large effect on the disruption kinetics and undisrupted cells after 15 minutes of ultrasonication. In particular, cells in stationary growth stage were more resistant to disruption by ultrasonication (fig. 5.7). This increase in resistance to cell wall disruption could be attributed to an increase in cell wall thickness and correlated well with cell wall thickness irrespectively of species or growth stage. The production of algaenan, however, did not seem to have a major effect on cell disruption kinetics for the species tested. Our data suggests that the mechanical strength of algaenan may not be much different than that of more common, polysaccharide based, walls within the green algae.

From an ecophysiological perspective, the thickening of the cell wall in stationary growth stage is one of many algal adaptations intended to increase the resistance of the cell to a number of environmental stresses such as predation, infection, desiccation etc. Furthermore, our finding that algaenan

does not significantly increase the mechanical strength of these cells makes sense for a number of reasons. Algae live in a relatively low-shear environment; that is they are not subjected to extreme mechanical forces so there is no selection pressure to increase mechanical strength of cells above those of polysaccharide cell walls. Furthermore algaenan producing algae generally divide by producing autospores which have to break the mother cell wall open to be release as algaenan cannot be broken down enzymatically. This may impose an upper limit to tensile strength above which it is too energy intensive to break the mother cell wall.

From an industrial perspective our results suggest that, if cells are to be disrupted, the point of harvesting is an important decision which may significantly impact the cost and/or efficiency of the downstream processing of algal biomass. Specifically gains in lipid content may be offset by the higher energy/longer extraction times needed to extract it. Therefore we propose that in the biorefinery model where cell disruption is necessary to extract high-value compounds, cells should be harvested around the end of the exponential phase. This would ensure maximum biomass productivity and good disruptibility of the cells. We also propose that algae considered for this approach should be scrutinised for high lipid content, lipid profiles and the production of value-added compounds in the exponential phase only. Moreover, published studies reporting on these aspects should also report the growth phase at which algae were harvested to aid correct species selection for each purpose.

Since no industrial scale disruption method has yet been realised in the emerging algal product industry and life-cycle studies are based on largely hypothetical data when considering cell disruption, it is impossible to know how growth stage would affect processing costs. Our data suggests that it could be an important, and so far underappreciated, factor with regards to algal downstream processing costs.

5.4.3 *The algal cell wall as a barrier to lipid extraction and implications for algaenan ecophysiology.*

We performed an exhaustive room temperature, sequential lipid extraction of six species of microalgae and full fat soybean flour to determine how algaenan production as well as cell wall thickness cell size and lipid content affect the extractability of these species. We found that algaenan producers had the lowest extractabilities in the first 45 minutes (fig. 11). This suggests that algaenan, apart from being insoluble in organic solvents (Allard *et al.*, 1998), is also relatively impermeable to chloroform/methanol which is one of the most efficient solvent mixtures for extracting lipids from algae (Lee *et al.*, 1998). This suggests that algaenan-producing species are more resistant to solvent extraction than polysaccharide-walled species and therefore cell disruption before extraction is more essential for the former than for the later.

After 180 minutes of extraction we found that algaenan was no longer the best predictor of extractability. Instead cell wall thickness was the main predictor of extractability (r^2=0.83) and this correlation was further enhanced when we used CWthicknes Cell size^{-1} Lipid content^{-1} as the predictor (r^2=0.88). The fact that algaenan ceased to be the best predictor after 180 minutes of extraction suggests that chloroform/methanol eventually permeabilises algaenan, possibly by dissolving lipids loosely bound around the algaenan core of the wall and thereafter algaenan is no longer the rate-limiting barrier to diffusion. The fact that cell wall thickness as well as cell size and lipid content correlated reasonably well with extractability can be explained in the following way. By keeping conditions the same and only varying species, diffusion across the cell wall becomes the main rate limiting step for the extraction. Therefore, all else being equal, a thicker cell wall will make the extraction slower. As cell size increases (all else being equal including cell shape), more lipid is contained within a given thickness of cell wall and also there is less surface area enclosing a given amount of lipid, therefore as cell size increases lipid extraction becomes faster. In the same

manner as lipid content increases, more lipid is accessible within a given wall thickness and surface area so the extraction becomes faster.

Because chloroform/methanol is a polar/non-polar solvent mixture, it is capable of disrupting hydrogen bonds between polar/non-polar lipid complexes associated to proteins and therefore extract a higher proportion and spectrum of lipids from the cell including polar, membrane-bound lipids (Cooney *et al.*, 2009; Halim *et al.*, 2012). It remains to be seen whether this property is essential to permeabilising algaenan or whether a single non-polar solvent such as hexane would have the same effect.

Final lipid content as revealed by sequential lipid extraction was considerably higher than lipid content extracted from the same biomass using the Foch method and among the highest reported in the literature (Griffiths and Harrison, 2009). This is because the Folch method by using a relatively short extraction time and mild conditions tends to underestimate the lipid content (Cooney *et al.*, 2009) and also because we grew the algae for a relatively long time period (21 days) meaning they underwent few to several days of nitrogen starvation which is known to increase the lipid content.

In section 1.4.2 we showed that there were significant differences between late exponential and stationary stage algae in their cell disruption kinetics. We did not perform lipid extraction in late exponential stage algae because we were interested mainly in elucidating the permeability of algaenan to solvents and this effect would likely be confounded by extracting lipid from actively dividing cells as these have often not completely formed algaenan walls (Atkinson *et al.*, 1972). However, given the above fact and the role that cell wall thickness plays in lipid extraction kinetics we expect that late exponential stage algae would also show different extraction kinetics than their stationary stage counterparts and that this may be partly offset by the lower lipid content and smaller cell size of the above.

We chose to also include soybean full fat flour as a comparison to a terrestrial biodiesel feedstock to test the claim found in some life-cycle

analyses that algal lipid extraction can be modelled in the same way as soybean extraction. Soybean flour was the most easily extractable material in our study (96.8 % of final lipid). Because soybean flour is finely ground, some cells will inevitably have been disrupted making the lipid more easily available to the solvent. In this case lipid extraction is a function of two processes; diffusion through the cell wall and washing of lipid from disrupted cells (McConnell and Farag, 2013). This would make lipid extraction for soybean faster even if the cell wall was the same as algae. Furthermore, the algal cell wall is the unit of interaction with the environment and the main protective barrier as in soybean the main protective barrier is the seed coat, so the cell walls of individual cells do not need to be as strong. Apart from the differences in cell wall ecophysiology, it has been recognised by experts in lipid extraction that downstream processes developed for soybean extraction would not be suitable for algae (Cooney *et al.*, 2009). Because new methods will need to be developed for microalgal extraction and these are still in their infancy it is at the present moment very difficult to predict how much energy will be required for algal lipid extraction and as our data shows, the burden of extraction on the algal biofuel life cycle will likely be species-dependent.

Our results support the hypothesis that the main function of algaenan is resistance to dessication. This is suppored by the fact that algaenan has, so far, only been found in freshwater or marine coastal species, that is species which would occasionally encounter dessication stress and furthermore would benefit by surviving seasonal drying of ponds and being transported on animals between watering points to spread to different ecosystems (see discussion of Kodner *et al.*, 2009). However the benefit of algaenan cell walls in surviving dessication has never been experimentally demonstrated.

Our results, while clearly highlighting the importance of the cell wall in downstream processing should be viewed with caution because so little is known about the physiology of the algal cell wall. As the interface between the cell and its environment, it is extremely important, and therefore algaenan production as well as other aspects of cell wall physiology may be

under strict control by the cell and thus could be variable even within the same species depending on media and environmental conditions. It currently takes an exhaustive chemical extraction procedure to prove the presence of algaenan in a species and it would take the same process to prove the absence of algaenan (Allard *et al.*, 1998). At present, the development of rapid and reliable techniques to detect the presence or absence of algaenan is absolutely essential. Furthermore algaenan has been defined from a geochemical perspective only. It has been shown that some algae possess tri-laminar cell walls, similar in appearance to algaenan but that do not satisfy the chemical tests to be considered such (Allard and Templier, 2000; Allard *et al.*, 1998; Kodner *et al.*, 2009). Therefore, from an ecophysiological as well as an applied industrial perspective it may be more logical to extend the term to any microalgae with highly aliphatic, "waxy/bioplastic", impermeable cell wall or coin a new term to include those cell walls.

5.4.4 Hydrothermal liquefaction.

We performed HTL of lyophilised, stationary stage (day 21) biomass from six species of green microalgae at 350 °C for 15 minutes. We obtained biocrude yields that were in accordance with the published literature for lipid-rich strains (Barreiro *et al.*, 2013a) although we found that the biocrude yield was in most cases less than the original lipid content as revealed by sequential lipid extraction. The solid fraction was also much higher than the ash content and similar to the highest solid yields reported in the literature (Barreiro *et al.*, 2013b; Biller *et al.*, 2012; Jazrawi *et al.*, 2013). This indicates a sub-optimal HTL protocol meaning that the algal biomass was not completely degraded. The low oil yields could also be a result of volatiles and other molecules being gasified through HTL and thus lost from the liquid phase. Gasified carbon, however should not be seen as completely lost energy as it can be burned along with conventional natural gas to recover the residual energy of the gas phase (Liu *et al.*, 2013). The fact that HTL showed suboptimal performance even though similar methods and species have

shown much lower solids content in the past (Biller *et al.*, 2012) may suggest that the growth of algae well into stationary stage (21 days) was the main deciding factor in obtaining such high solid contents, however our experiments unfortunately did not control for growth stage. Because the species tested in the present study showed thicker and more resistant cell walls in stationary stage, it is possible that HTL would need additional optimisation for stationary stage algae such as higher temperature and/or higher retention times.

HTL experiments also showed some of the lowest nitrogen and sulphur contents reported in the literature. This low N content of the biocrude oil should be attributed to letting the algae grow into stationary phase and thus undergo nutrient starvation. In stationary phase the C/N, C/P and C/S ratios are increased because the cell is limited for many functions such as protein and DNA synthesis required for cell division but is still able to fix carbon through photosynthesis. Algal cells tend to accumulate carbon compounds such as carbohydrates (commonly starch) and lipids to use as energy sources when conditions become favourable for cell division. In terms of HTL this means a reduced content of N and S in the biomass leading to a reduced content of these compounds in the biocrude. Nutrient starvation is not an optimal strategy for lipid accumulation because it reduces the biomass and lipid productivity of the whole facility. However when combined with the energy credit of a reduced need for upgrading the oil and increased efficiency of nutrient recycling, having species go under mild nutrient starvation or choosing species with an inherently low nitrogen content, may become an interesting strategy for optimising HTL. However this hypothesis would need further experiments and a life-cycle analysis to be properly evaluated.

5.4.5 Summary and concluding remarks.

Our study, by using a six-species screen of green microalgae has shown that anaerobic digestate in appropriate dilutions is a good medium for algal

growth, whether sterile or non-sterile. Secondary treated municipal wastewater, on the other hand, is a satisfactory medium in its sterile form but very challenging to grow algae in its non-sterile form. Some algae where able to grow and dominate non-sterile WW (especially *C. emersonii*) while others disappeared from the medium (*C. vulgaris*). The only two algae that showed good growth rates in both sterile and non-sterile WW were algaenan producers but also had the slowest growth rates. Predator/prey and allelopathic interactions can be very complicated and as such we cannot conclude that producing an algaenan cell wall limits the infection/predation potential of microalgae.

Ultrasonication experiments for cell disruption showed that algaenan production did not significantly contribute to increased resistance to cell disruption, however growth stage significantly reduced the disruptibility of cells. We found this was most likely due to an increase in cell wall thickness that takes place in the stationary phase of growth. We also found that the only non-coccoid alga (*P. ellipsoidea*) was the most easily disrupted among our species and this could not be attributed to cell wall thickness. Therefore having a non-coccoid shape may make disruption more economical but our experiments were not designed to control for this so further research is necessary.

Algaenan did have a significant effect on the extraction kinetics of undisrupted cells. Algaenan-producers showed the slowest extractions in the first 45 minutes. However by 180 minutes extraction kinetics were not limited by the production of algaenan but rather were inversely proportional to cell wall thickness and proportional to cell volume and lipid content. This suggests that cell wall composition and thickness are the main rate-limiting steps in solvent extraction of lipids from undisrupted cells.

Hydrothermal liquefaction of stationary stage algae showed high solid and low biocrude yields suggesting that this process needed further optimisation. This is possibly due to stationary stage algae being more resistant to HTL degradation than exponential phase algae which have been

the majority of specimens studied so far. However our stationary stage species also showed some of the lowest nitrogen and sulphur contents so far reported. This is beneficial because it would reduce the cost of oil upgrading and increase the efficiency of nitrogen recycling, leading to reduced need for importing nutrients such as inorganic fertilisers or anaerobic digestate.

Growth stage of algae seems to be of major and underappreciated importance when considering downstream processing costs, however it is rarely reported in studies concerning the downstream processing of microalgae. We invite researchers hereafter to explicitly report in their methods not only how many days the algae were grown for but also at which growth stage they are harvested. This would greatly facilitate the correct interpretation of experimental results on the efficiency of downstream processes.

The algal cell wall is of major importance when considering the costs of downstream processing. We found that cell wall thickness was important in most cases and that the production of algaenan was limiting only in the case of solvent extraction from undisrupted cells. This supports the hypothesis that the main function of algaenan in the wild is resistance to dessication.

In terms of the biorefinery concept our results suggest that the best species might be one with fast growth rates, high lipid content in the exponential stage, a thin cell wall and a non-coccoid shape. The presence of algaenan-walled species would only be acceptable if a cost- and energy-efficient, industrial cell disruption method becomes available. Otherwise algaenan-producing species would best be avoided. In a biorefinery concept it would be most beneficial to harvest algae at the late exponential stage to ensure maximum biomass productivity and to minimise the cost of cell disruption. We envision a fully integrated algal growth facility where anaerobic digestion would be a suitable final processing step for the extracted biomass to obtain methane for the electricity needs of the plant and CO_2 to support algal growth as well as digestate to use as a growth medium to close the nutrient loop in algal growth facilities. Organic solid waste would be

imported to co-digest with the algae in order to supplement for inherent nutrient losses in the system. To further develop the biorefinery scenario, future research is essential in developing a scalable and efficient cell disruption method, further elucidating the role of the cell wall in determining disruptibility, extractability and digestibility of algal species in order to better inform species selection and finally in optimising the nutrient recycling by anaerobic digestion and co-digestion of several species of microalgae, in several states (whole, disrupted, extracted) and with several other agricultural or food industry solid wastes.

From an HTL perspective our data suggests that growth stage may be important in reducing the N and S content of the biocrude. This may come at a cost since stationary stage algae may need more energy to process (higher temperature and/or residence time). Much work is still needed to optimise HTL from an EROI perspective but we have highlighted some new opportunities for optimisation. The issue of nutrient recycling may be more complicated with HTL than with AD but co-liquefaction with solid waste would clearly be an option worth researching in order to close the nutrient loop and replace the lost nitrogen at the minimum possible energetic and environmental cost. Therefore the most essential future research concerning HTL is optimisation of integration; growing various species or consortia in various conditions, optimising HTL for each of those and co-liquefying with various sources of waste nutrients, recycling the aqueous phase for algal growth, assessing biocrude yields and properties and evaluating the results from a life-cycle perspective.

Our impression from carrying out this research is that the biorefinery concept may still need significant engineering advances to become viable for algae but eventually it will be able to extract more value (and potentially profit) from the algal biomass. However simple HTL for biocrude can be significantly integrated and optimised with a few simple steps within the next 5 years.

As a final point we have shown that cell wall physiology, as it varies between species and also between growth stages of the same species, is an important consideration in the downstream processing of microalgae. Therefore we urge researchers to further elucidate the role of the cell wall in the above processes and to report thoroughly on the growth regimes and point of harvesting of their algal cultures as we have shown that this likely is of major importance in downstream processes such as lipid extraction, cell disruption and HTL. Discrepancies in growth conditions between different research groups may well be important in explaining their differing results from similar experiments.

6 General discussion and personal reflections.

6.1 The dawn of a new agricultural revolution.

Algae represent a huge untapped potential for the production of energy, food and for the bioremediation of various wastewaters (Abdel-Raouf *et al.*, 2012; Chisti *et al.*, 2011; Craggs *et al.*, 2012; Mata *et al.*, 2010; Packer, 2009). There is no doubt that algae have a potential role to play minimising human footprint in terms of atmospheric CO_2 and eutrophication while providing energy in the form of liquid biofuels and biogas, food as well as high-value food supplements and pharmaceuticals (Milledge, 2010). The main hurdles currently limiting the utilisation of algae are those of the scaling of downstream processing and especially cell disruption, initial capital investment against uncertainty and system integration (Greenwell *et al.*, 2010; Lam and Lee, 2012; Scott *et al.*, 2010; Singh *et al.*, 2011). Algal growth systems need to be fully integrated in order to regenerate and recycle nutrients and CO_2 as well as efficiently utilising electricity produced and waste heat on site. Algae also have the potential of increasing global productivity by utilising desert locations, however water availability and/or recycling can be an issue. In the very long term the main disadvantage of algae will be that the systems need a high capital investment and considerable amounts of machinery and expertise to run efficiently so the culturing of algae is unlikely to ever become a true agricultural revolution. Research and development after decades of high promises and low performance seem to be tantalisingly close to achieving algal culturing at truly industrial scales.

6.2 The role of species selection, genetic modification and artificial ecology in the future of microalgae.

A great number of species of algae have been described and assessed so far for industrial applications (Nascimento *et al.*, 2012; Sheehan *et al.*, 1998) but a large untapped diversity still exists out there as algae are ubiquitous in almost all environments. Therefore rigorous bioprospecting should continue to be a high priority among microalgal producers. Large gaps in our knowledge of the ecology, life-cycles and ecophysiology of algae still exist and while these may seem of minor importance to the algal biofuel research community, they need to be better understood in order to optimise performance not only in growth and lipid production but also in downstream processing. Furthermore, the sheer volume of cultures needed for any industrially meaningful production means that sterility cannot be maintained and release to the environment is unavoidable. Therefore algal cultures should be treated and understood as communities and we urge more research to take place on the co-culturing of different species of algae and other microorganisms such as yeasts, fungi and bacteria in order to create stable communities that perform in a predictable manner while filling all ecological niches to limit the potential for contamination and culture crashes. We believe future algal growers should be rigorously trained in microbial ecology and that the interaction between biofuel researchers and ecologists seems to be currently lacking.

One way of avoiding the complexities of creating and monitoring an artificial community is to culture extremophilic algae. These would be organisms that may persist in the wild albeit in very limited habitats or showing very slow growth rates in normal conditions. The acidophilic alga *Pseudochoricystis ellipsoidea* (Satoh *et al.*, 2010) is one of the best examples of such a species as it can adapt to normal pH levels but at suboptimal growth rates. Extremophilic algae have great potential for the specific culturing of single species and more research and bioprospecting on this subject could be greatly beneficial.

Extremophilic algae such as *P. ellipsoidea* are good candidates for genetic modification because their release into the wild has fewer potential consequences. Genetic modification of microalgae is still at an early stage of development but rapid progress is being made (Radakovits *et al.*, 2010). Algal GM has to be done in a strictly ecological context (Flynn *et al.*, 2012). This context is perhaps more important in microalgae than any other organism because microalgae can be seen as the basis of all life on earth. Because it is challenging to exclude the main natural competitors and predators regardless of the culture system, algae must be engineered in such a manner that they are capable of remaining the top competitor in an open ecosystem while posing no threat to natural aquatic communities. We believe this should firmly exclude the production of drop-in fuels that are toxic to or non-utilisable by zooplankton and fish because it would be very easy for these algae to colonise a natural body of water and kill or drive off most other diversity of life. Flynn *et al.* (2012) concluded that even the overexpression of lipids would make engineered algae a poor food source for zooplankton so this modification should also be considered potentially harmful. Algae feed most of the planet so we should not be trying to make them a poor food source in order to fuel our transport otherwise we are not improving on the current situation where we have people starving and pristine land converted to fuel crops in order to fuel our transport.

The best genetic modification that could be made in algae would be the improvement of the efficiency and productivity of photosynthesis (Melis, 2009; Stephenson *et al.*, 2011). Photosynthesis and carbon capture are ultimately the engines driving all productivity on this planet and better photosynthesis and carbon capture would mean more life or at least a more economical human utilisation of nature. This has been achieved in shortening the light harvesting antenna of algae meaning that the rates of light harvesting and carbon capture are better matching under high light and thus light is used more efficiently, cultures can be denser and more productive. Unfortunately though light levels below those required to saturate photosynthesis will favour an increased production of chlorophyll and thus cells will always tend

to adapt towards high chlorophyll unless they are constantly kept at a light intensity high enough to saturate photosynthesis. Regardless of the long-term stability of this modification it is clearly one worth pursuing because of its huge potential and its highly reduced fitness in the wild.

6.3 The potential of *Pseudochoricystis ellipsoidea* for biofuel production.

The vast majority of this study focused on culturing, improving and comparing the performance of *P. ellipsoidea* to other species of microalgae. We found this species to be fast growing with good biomass and lipid productivities as well as being highly resistant to contamination and a top competitor even in non-sterile wastewater at neutral pH. Furthermore and perhaps most importantly it was by far the most easily disruptable of the six species studied even while producing an algaenan cell wall. It is somewhat ironic that this was only discovered after two years of trying to modify this cell wall and that the main reason for the disruptability of this species was probably its shape which was a factor not tested in this study. However it is important to state that the methods developed here for the mutagenesis and high-throughput screening for cell wall mutants will be useful for this and other species of algae and we would be very happy to see researchers develop them further and apply them to other species with the target of inhibiting algaenan formation and understanding algaenan biosynthesis. In conclusion, *P. ellipsoidea* due to its growth and lipid productivity, disruptability and resistance to contamination is certainly one of the species with the highest potential for biofuel production on an industrial scale.

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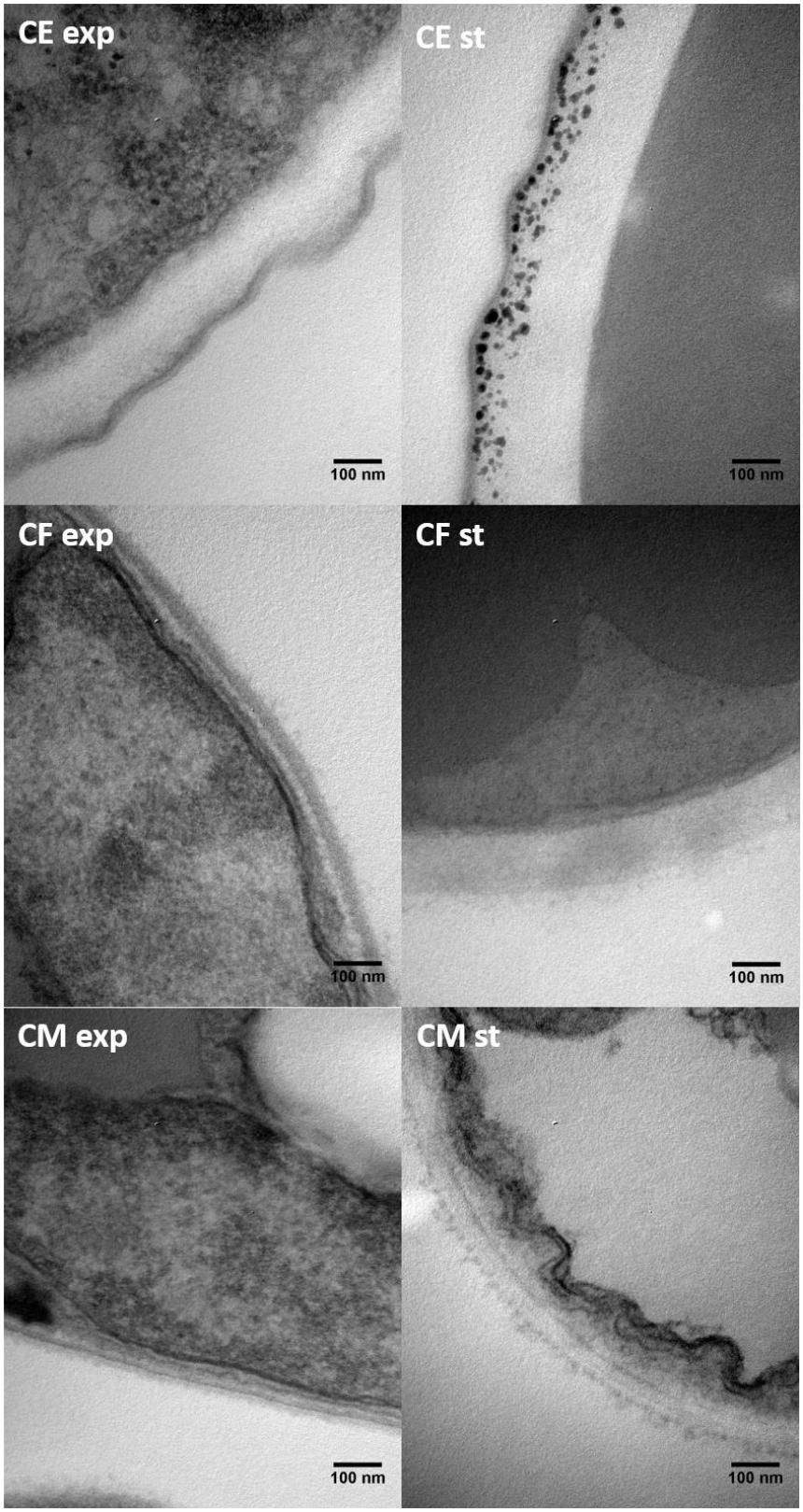
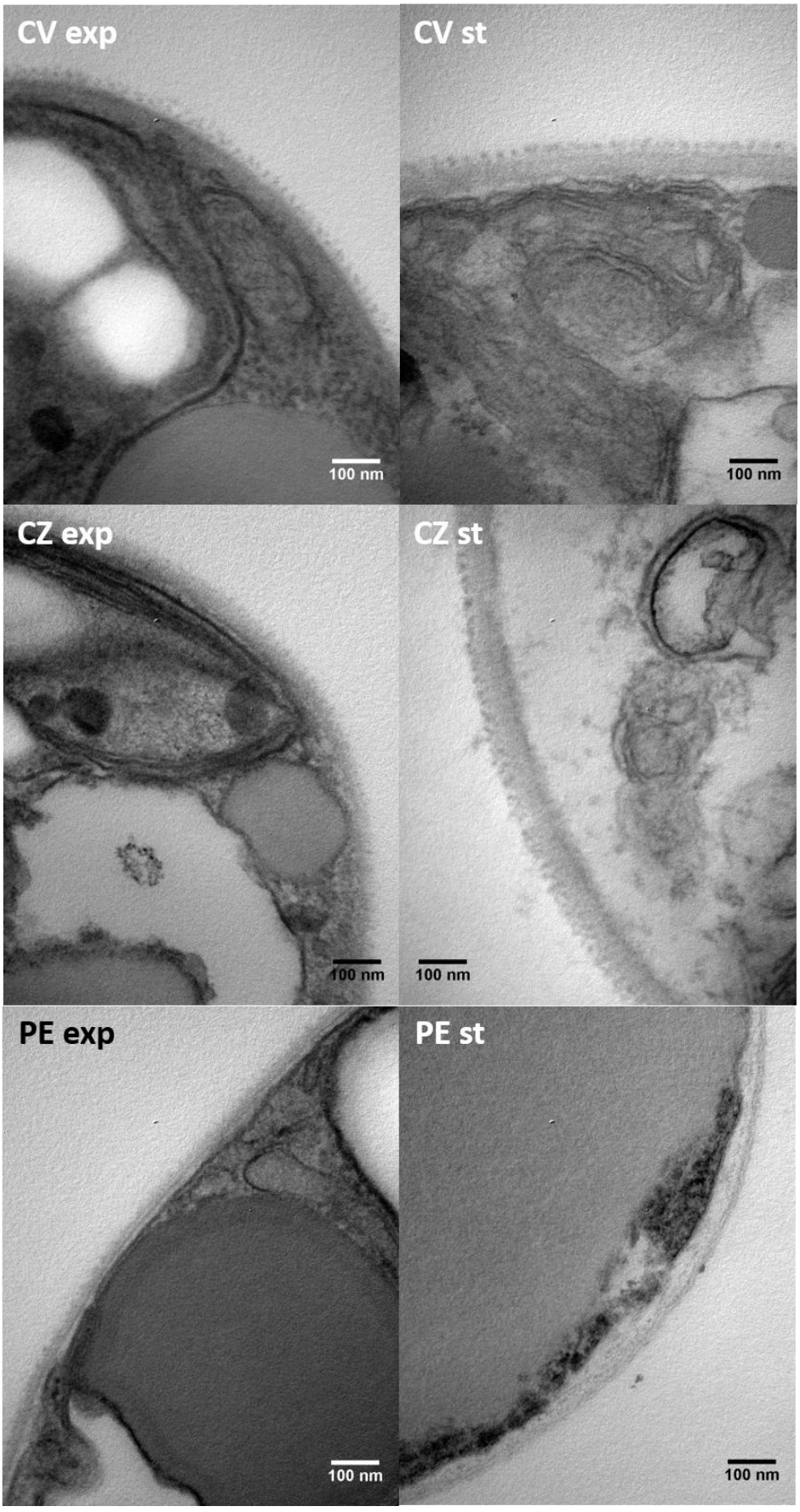
8 Appendix

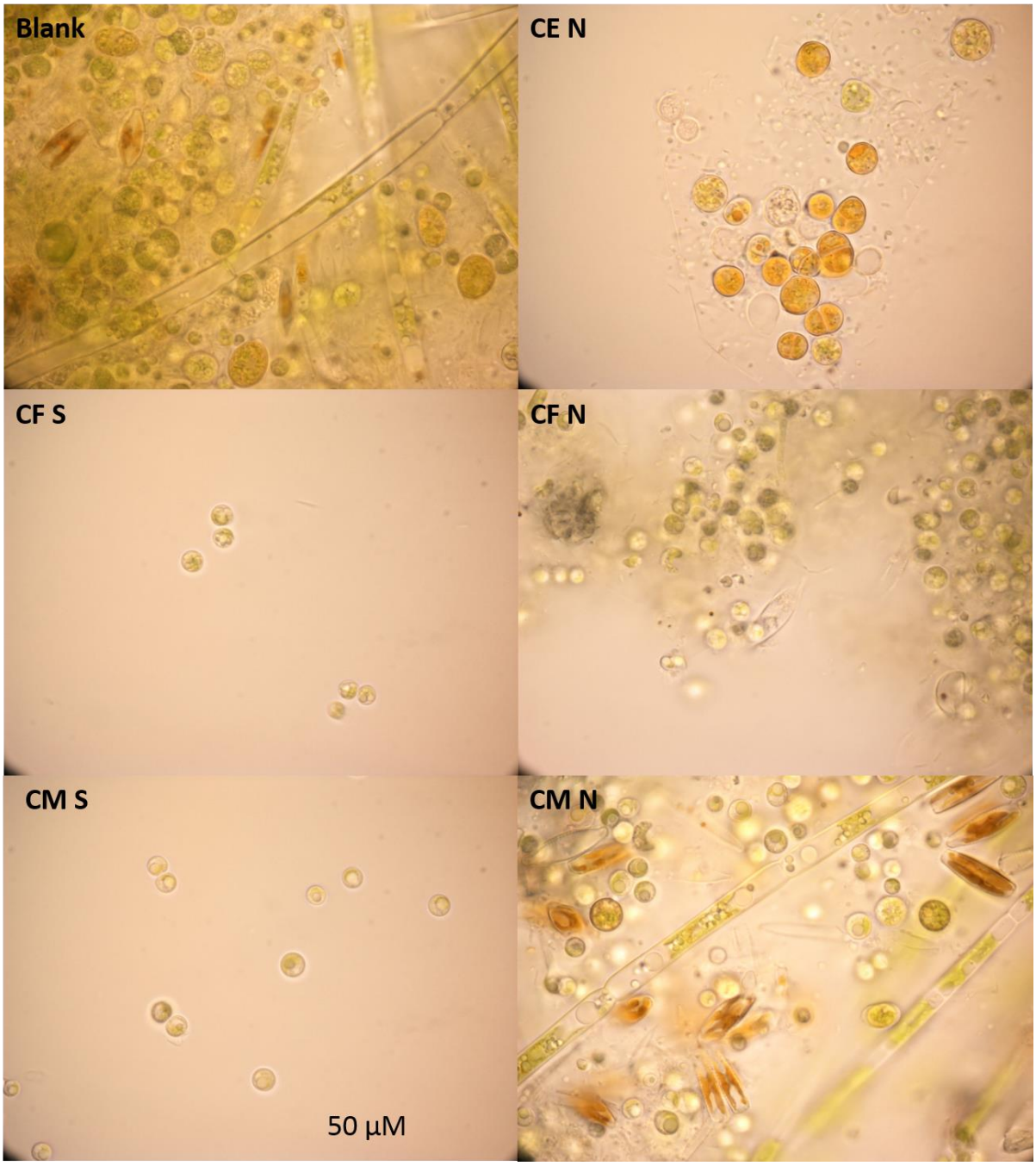
8.1 Transmission Electron Microscopy of six species of microalgae.

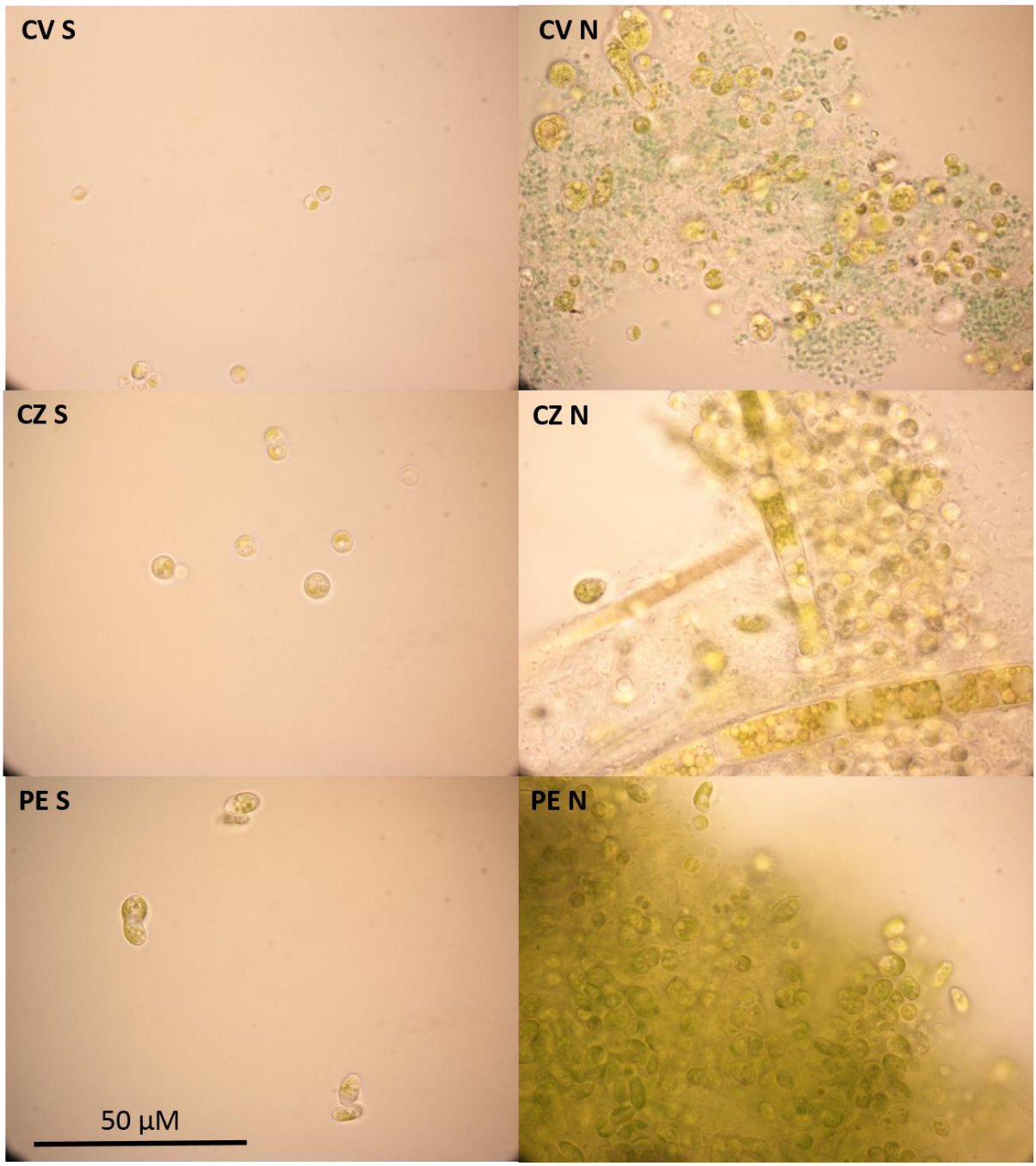




Initials denote species (see abreviations) "exp" denotes exponential stage and "st" denotes stationary stage.

8.2 Light microscopy of autoclaved and non-autoclaved wastewater for six species of algae (day 21).





Light microscopy of six species of microalgae in autoclaved (left) and non-autoclaved wastewater. Initials denote species (see abreviations) S=sterile, N=non-sterile. Note the absence of other species in non-autoclaved CE and the dominance of cyanobacteria in non-autoclaved CV.